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FORM AND CAUSALITY IN NEUROGENESIS

By JEAN PIATT
Department of Anatomy, University of Pennsylvania, U.S.A.

(*Received* 10 *July* 1947)

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I. INTRODUCTION

This review will attempt to bring together those experiments and observations which have some bearing on the many specific problems relating to the causality of form within the embryonic central nervous system. In the abstract the subject-matter of such a study should possess a homogeneity in both material and approach; actually, this has not been the case. There is no general cadre or methodology of approach serving to guide studies of this nature into the most fruitful channels. The field of causal neurogenesis lies in a kind of biological no-man's-land between neurology and experimental embryology and must draw upon one or both of these well-grounded disciplines for its substance. It has no true historical background; its genealogy is spurious. To put it rather fancifully, causal neurogenesis has been the female partner in a sort of morganatic marriage, the issue of which has been without recognition or title. Neurology, by and large, has not concerned itself over problems of structural causality. Experimental embryology has had as its aim the solution of a number of related problems dealing with the origin of form. However, in the case of the central nervous system, there are relatively few studies aimed directly at such a goal. Many of the data have been indirect and ancillary in nature. Structural configuration within the nervous system has been used to interpret other phenomena, rather than to explain neurogenesis itself. The available data upon which to base the principles of causal neuroembryology are not, therefore, neatly circumscribed. The study of causal neurogenesis is a new and somewhat arbitrary scientific discipline, not so much in point of time or raw material as in the ends it seeks to attain. Because of the necessarily eclectic nature of the subject-matter, it has seemed advisable to present a broad, comprehensive review rather than any one particular phase of the problem.

This article will concern itself, as nearly as possible, with the causal factors responsible for the structural development of the embryonic central nervous system. Reviews on the structural laws (Weiss, 1941*a*) and directional growth (Straus, 1946) of the peripheral nervous system have recently appeared. The problem of functional reorganization in the central nervous system has been adequately treated by Sperry (1945). It is difficult, of course, to analyse separately form and function in the development of the nervous system, and structural laws found to be operative in the central nervous system will undoubtedly, in many cases, play identical roles in the growth of the peripheral nervous system. The concepts of dependent differentiation and self-differentiation, as outlined by Roux (1885), are inherent in any such discussion, but, for the most part, the available data are inadequate for a proper evaluation of their respective roles in any given case. References are chiefly those dealing with amphibian and avian material, since amphibians and birds furnish by far the greatest bulk of the research data; the emphasis, for the most part, has been placed upon the relatively recent experimental approach.

II. EARLY NEUROGENETIC FORCES

(1) *Medullary plate and tube formation*

It has been recognized for some time that the earliest visible elaboration of the central nervous system (neural plate and folds) is dependent to some extent upon mesodermal substratum, or roof of the archenteron, the latter itself having previously formed the presumptive chorda-mesoderm or dorsal lip of the blastopore. The full significance of this so-called inductive effect was first pointed out by Spemann & Mangold (1924) in their now classical experiment. Following this, Marx (1925) made systematic transplantations of the presumptive medullary plate of *Triton* into the epidermal region of other early embryos. These presumptive plate grafts were made with and without the underlying mesoderm and demonstrated that neural tissue was formed only after contact with the archenteric roof had been established. Lehmann (1926, 1928) made various defect experiments in the dorsal lip of *Triton* embryos in all stages of gastrulation and came to the conclusion that the resulting abnormalities in the medullary plate and tube showed that the mesodermal substratum is responsible for normal nervous system formation. He believed that neural tube formation was due to two factors: (1) a labile determination in the neural plate ectoderm itself; (2) the determining factor inherent in the mesoderm. Mangold (1929) further showed that medullary plate and brain tissue from tail-bud stages placed in the blastocoel cavity of gastrulae were themselves able to induce neural tissue in the presumptive epidermis of the host embryos. Brain tissue from 2 cm. larvae lacked this inductive capacity. Again the primary inductive action of the chorda-mesoderm was demonstrated, as well as the secondary organizing capacity of early neural tissue itself. This matter of 'secondary organizer' was shown clearly by the experiments of Spemann & Geinitz (1927). Pieces of ventral ectoderm from early *Triton* gastrulae were implanted immediately above the dorsal lip of the very early gastrula. After invagination of the transplant it was reclaimed and placed in the blastocoel cavity where it induced the formation of well-developed neural plates in these latter hosts. The process of gastrulation and its attendant phenomena had imparted to prospective epidermal tissue the power of neural organization. The later work of Eakin (1933) showed that when the roof of the archenteron was mechanically prevented from coming into contact with presumptive neural ectoderm, no dorsal medullary plate was formed; on the contrary the ventrally displaced chorda-mesoderm coming into contact with the vegetative area caused a ventral neural plate to develop. Finally, Goerttler (1927) claimed that dorsal lip material was not equipotential and that good differentiation of neural tissue from transplanted presumptive plate occurred only when the tissue developed in what he termed a 'favourable environment'. Holtfreter (1933*a*, *c*) repeated some of Goerttler's experiments and arrived at the conclusion that neither position (Goerttler's 'favourable position'), nor prospective significance nor developmental stage of host (within the limits of his experiments) have any influence on frequency of medullary differentiation.

It is of interest to note that prior to the experiments just mentioned, Lewis (1906*a*, 1907), working on dorsal lip transplantation in *Rana*, actually 'discovered' the organizing capacity of this tissue. Lewis observed that his transplants developed into neural tissue (as well as other types) but simply concluded that nervous tissue is predetermined at an early stage (gastrula). He did not consider that neural tubes which developed in the region of his grafts were 'induced' from host tissue owing to the proximity of the ectopic dorsal lip. As pointed out by Needham (1942), the neural tubes observed by Lewis may have resulted from a process of self-differentiation of the donor tissue, since the host tissue was too old (tail-bud) to respond to an inductive effect. However, since dorsal lip itself is not presumptive nervous tissue, it is difficult to see how such tissue could self-differentiate into neural tubes. Regardless of what interpretations are made concerning the early experiments of Lewis, they probably represent the first account of the primary organizing capacity of dorsal lip and prospective chorda-mesoderm and its relations to early nervous tissue formation. The problem of induction and organizers *per se* is beyond the scope of this review (cf. Mangold, 1928; Spemann, 1938; Needham, 1942).

More recent experiments by Barth (1939, 1941) have thrown some doubt upon the importance of the organizer as the causal agent in neural plate differentiation. Earlier experiments on *Triturus* by Schechtman (1934) had indicated that development of a normal medullary plate involved factors inherent in the presumptive plate material itself, as well as inductive action of the mesoderm. In a following paper Schechtman (1935) modified this conclusion and decided that the archenteric roof was the all-important factor. Barth placed explants of gastrula ectoderm without mesodermal substratum into salt solution and found that under certain conditions neural tubes were formed out of these isolated pieces. He concluded that not only was the mesoderm unnecessary for neural tube formation but that an anterior-posterior gradient was inherent in the presumptive neural ectoderm. Since Barth's explants had never come in contact with chorda-mesoderm, the neural gradient cannot be explained by regional differences in the organizer material (Hall, 1932).

The explantation experiments of Barth are contrary to earlier work along similar lines performed by Holtfreter (1931*b*, 1933*b*). Recent investigations by Holtfreter (1944, 1945) have extended his former work and in the light of these later results he has re-evaluated the conclusions of Barth. Holtfreter found that in the species used by Barth (*Amblystoma*) the uncoated inner ectodermal cells of the presumptive medullary plate explants are injured by exposure to the salt medium and thinks that the autolysis of these cells liberates a neurogenetic substance which is the real inductor. He concludes that the underlying inductor tissue is still the agent causing neural plate formation and that the limitation of self-organizing power of gastrula ectoderm is well established. Holtfreter also believes that cell autolysis, resulting in a liberation of toxic neurogenetic substances, is the probable explanation for the wide variety of neural inductions obtained with different chemical and dead substances. A report on the neurogenetic properties of non-living substances and the effect, both direct and indirect, of chemicals on inductor and neural tissues is beyond the scope of this review. More recent work is that of Hall (1942), Shen (1942) and Ranzi (1945). The search for the chemical nature of the organizer continues, but as Sinnott (1946) remarks, 'No analysis, however complete, of substances known to have morphogenetic effects will explain their results. Only a knowledge of the organized system upon which they act will solve the problem.'

The actual mechanism of neural plate folding and the physical and biological principles involved in the formation of a hollow tube from a flat neural plate are still poorly understood. Some of the earliest work in experimental embryology was directed toward this end. It was shown, for instance, that the folding process of the neural plate was not due to pressure exerted by the adjacent non-neural tissues. With this in mind Glaser (1914, 1916) made the first real analysis of neural tube formation. Glaser concluded that there was a differential water absorption by the elongated cells of the neural plate, whereby the inner ends of the cells took up more water than the outer ends. This, Glaser thought, would result in a weakening of the inner (deeper) surface of the plate and its involution into a tube. Boerema (1929) also noticed that cells of the neural plate, at first cylindrical, later became wedge-shaped (broader at their inner ends) and suggested that this change of shape would necessitate a bending of the plate inward upon itself. Both Glaser's and Boerema's explanations are essentially mechanical in nature and serve to emphasize that plate folding is neither a pushing nor a pulling of extra-plate material. The plate is not passive.

More recent work has added further data concerning details of neural plate movements and folding but no generally accepted explanation for the entire process has been forthcoming. Brown, Hamburger & Schmitt (1941) found little difference (0·003) in the specific gravity between flat and folded neural plate of neurulae of *Amblystoma* and *Rana*. They interpret this negligible change in density as indicating only a very slight change in water content of neural tissue during formation of the neural tube. This conclusion is in direct opposition to the cardinal point of Glaser's thesis. The explanation they suggest is extremely hypothetical and embodies the principle of attractive forces between surface molecules of cells. Hutchinson (1940, 1944) found a slight decrease in number of cells per unit volume of neural plate between stages 13 and 16 of *Amblystoma*, and Burt (1943), using the same species, concluded that neurulation is accompanied by mitosis but that the mitotic rate rises only after closure of the neural folds. Finally, Gillette (1944) observed that during early neurulation neither differential mitotic activity nor increase in cell size occurred. From the results of these last three investigations it would appear that, although neurulation may be autonomous, changes neither in cell number nor changes in size can account for the process. Lewis (1947) believes that the essential factors for invagination or folding of the neural plate are either an increase in the contractile tension of the superficial gel layer of the external ends of the plate cells or a corresponding decrease in contractile tension of the internal ends. Lewis agrees with Brown, Hamburger and Schmitt, and Gillette that Glaser's idea of plate folding due to enlargement and hydration is wrong.

Important confirmation of the dissociability between neural tube formation and alterations in cell number and volume comes from irradiation experiments on early chick embryos. Hinrichs (1927) found that ultra-violet radiation did not affect the ability of neural plate cells to divide and proliferate but that in many cases it did inhibit the closure of the medullary plate. Davis (1944) observed that ultra-violet radiation of wave-lengths 2483–3130 Å inhibited the folding process of neural tube formation while cell division and volume changes continued undisturbed. It is interesting to note that cell division in the experiments of Davis was uninterrupted, since Frank & Gurwitsch (1927) report that with ultra-violet wave-lengths of 2700 Å (within the range used by Davis) mitosis in the onion root tip was not produced.

Experiments by Roach (1945) are of great interest in throwing light upon the determination of the various axes of the early central nervous system, these axes themselves being the pristine expression of the regional differentiation of the brain into its specific parts.[2] Roach reversed the antero-posterior axis of both whole and unilateral portions of the medullary plate, stages 13 and 14 in *Amblystoma*. Medio-lateral reversals were also made. She found that regardless of whether mesodermal substratum was reversed with the ectodermal tissue or simply left in place the brain parts of the operated larvae were completely reversed in the antero-posterior reversal experiments. This was true in both whole and unilateral reversals. In the case of medio-lateral reversals the larval brains were found to be symmetrical and bilaterally similar. In other words, the antero-posterior axis of the future central nervous system is already determined in the preneurula stage (13) and the underlying mesoderm no longer exerts any influence upon its subsequent morphological gradient, while the medio-lateral axis is not yet polarized even in the early neurula stage (14) in either the medullary ectoderm or the mesodermal substratum.

Reversal of the medullary plate was first performed by Spemann (1906, 1912). Spemann also found that the pattern of the brain parts was completely reversed following antero-posterior rotation of the medullary plate. In Spemann's experiments both mesodermal substratum and ectoderm were always reversed together and the development *herkunftsgemäss* has usually been considered as due to this fact (Mangold, 1928). Adelmann (1929*a*, *b*, 1930) concluded also that medullary ectoderm alone was incapable of normal differentiation and that the mesodermal substratum in urodeles exerts a formative influence on specific parts of the brain. Alderman (1935), working on neurulae of *Hyla*, obtained a normal (*ortsgemäss*) sequence of brain parts following 180° rotation of median squares of the extreme anterior end of the medullary ectoderm. Ectopic transplants developed into specific regions of the brain only when underlain by mesoderm. Whenever any considerable amount of brain tissue developed in these heterotopic grafts, Alderman usually allocated it as diencephalon. This seems rather strange since the extreme anterior end of the medullary plate normally forms part of the telencephalon. From Alderman's figures it would seem that some of these are actually telencephalon median cases.

The experiments of Roach appear to be thorough and systematic and indicate that mesodermal substratum is less important in regulating the *A–P* axis than previously thought. Hutchinson (1936) also showed that regulation of the medio-lateral axis of the future brachial region of the medullary plate in *Amblystoma* is independent of the mesodermal substratum. Although his results indicated that by stage 15 the medio-lateral axis is determined, he observed complete and early regulation into perfect neural tubes in many cases. Roach's data are further supported by Burch (1946), who found that 180° rotation of the medullary plate of *Hyla* (same species used by Alderman) resulted in a reversed infundibular depression in the floor of the rhombencephalon. A regional *A–P* gradient also exists in very early embryos of the chick (Dalton, 1935). Roach is careful to point out that the influence of the substratum is not actually eliminated, since even at stage 13 in *Amblystoma* the ectodermal part of the plate is underlain by mesoderm. Her medio-lateral reversals, however, give no support to the influence of the mesoderm in determining this particular axis; normal brain regulation occurred despite the inclusion of rotated substratum.

Whatever may eventually prove to be the exact influence of the mesodermal substratum on the morphological axes of the early central nervous system and the pattern of its definitive regions, more recent experimental data do not assign to the medullary ectoderm a completely passive role.

(2) *Segmentation*

Metamerism of the central nervous system has been observed and described by numerous workers. The morphological significance and fate of the various neuromeres have been discussed and an attempt made to indicate their homologies from one species or group to the next. These investigations by earlier morphologists have been almost entirely descriptive in nature and have usually been analysed within the larger problem of head segmentation. The contradictory conclusions from these early studies and the almost complete lack of really critical or experimental work along these lines have resulted in an ignorance of the causal factors underlying neuromerism and the role they may conceivably play in the future elaboration of brain and spinal cord. Griggs (1910) came to the conclusion that in *Amblystoma* true neuromeres vary in number from one embryo to the next and are found only in the open medullary plate stage. He maintained that those swellings that occur later in the neural tube are not true neuromeres but are caused by secondary processes. Smith (1914) went even further and decided that the early foldings seen in the open medullary plate in urodeles are not constant and have no morphological significance but are simply due to the longitudinal stretching of the neural plate. He thought it unlikely that segmentation of the neural plate should precede that of the mesoderm. Coghill (1913) also thought that neuromeric organization of the cord is a secondary acquisition and Stockard (1921) refers to the early neural tube as an undifferentiated axis. Child (1921), however, advocated the primacy of ectodermal segmentation over that of the mesoderm and speaks of the spinal ganglion as the primary definitive organ of the segment.

Experiments begun by Lehmann (1927) have cast doubt upon the primacy of ectodermal segmentation. When several somites were removed from early embryos of *Pleurodeles*, Lehmann found that the corresponding spinal ganglia of that side were either completely absent or arranged in unsegmented strands. Furthermore, he showed that spinal ganglia will develop and segment properly only when adjacent to the medial surface of the somites; the lateral somitic surface was observed to inhibit ganglion formation. He concluded that spinal ganglia do not develop nor differentiate well unless somitic mesoderm is present and points out that his experimental data are not in accord with Child's account of the primitive segmentation of the neural ectoderm.

A series of investigations by Detwiler and his associates has extended Lehmann's work. Detwiler (1929*a*) agrees with Lehmann that removal of somites results in a suppression of ganglia but found that some ganglion cells are usually present, although not regularly arranged. Motor roots, though small, were always present and regularly placed. Removal of both segmented (stage 29) and non-segmented (stage 23) somitic mesoderm in *Amblystoma* (1932*a*) disturbs the development and orderly arrangement of the spinal ganglia but does not completely prevent either their formation or segmental arrangement. Removal of earlier (non-segmented) axial mesoderm causes somewhat more disturbance of the ganglia than does extirpation of segmented mesoderm.

This study also indicated that the lateral surface of the somite does not inhibit ganglion formation, as claimed by Lehmann, and showed that ganglia will still develop on the adjacent sides of host and donor cords in the absence of intervening mesoderm. Detwiler concluded that somites exert an early morphogenetic influence upon spinal ganglion formation but that this influence is neither essential nor an all-or-none affair. Additional work (1933*d*, 1934) showed that not only did the absence of mesoderm result in a ganglion deficiency but that a supernumerary number of somites for a given length of cord called forth an increase in the number of ganglia, sensory and motor roots in this region; although, when more than one additional somite was present, there existed no strict correspondence between total number of grafted somites and spinal nerves. Strips of unsegmented axial mesoderm, possessing potentialities for more somites than the replaced ones, showed a closer correspondence between supernumerary myotomes and ganglion number. These latter experiments offer even more convincing data than those in which axial mesoderm was simply extirpated. They strongly indicate that segmentation of ganglia and associated nerves is subservient to mesodermal metamerism and that an intrinsic metamerism of the spinal cord is either secondary or nonexistent.

Detwiler & Van Dyke (1934) have demonstrated that when the dorsal portion of the brachial cord region is extirpated, motor roots and nerves develop in orderly arrangement even in the absence of ganglia and sensory roots. They interpret this as an indication that the characteristic number of motor roots is, in general, conditioned by the myotomes and not the cord itself. Chase (1940) also found that absence of ganglia did not affect the number or size of the motor roots in most cases. Yntema (1943), however, has shown that deficiency in spinal ganglion size and number is often correlated with absence or reduction of motor root fibres. The segmental arrangement of the motor roots when present was usually normal. This developmental relationship between motor roots and somites is at slight odds with the earlier work of Detwiler (1929*a*) already mentioned. When post-brachial cord segments are transplanted heterotopically both with and without adjacent somites in *Amblystoma* of stage 29, Detwiler (1935) found that only on the side of the transplant having somites did the ganglia develop normally and in regular order. Ectopic cords without somites and notochord developed reduced ganglia which tended to lie dorsal to their normal position. This latter point would indicate that position as well as segmental arrangement is influenced by the mesoderm. Coghill (1914) had also noticed that Rohon-Beard cells in embryonic cords of *Amblystoma* were situated dorsally when the somites extended dorsally and more ventrally when they did not. Work by others (Yamane, 1930; Rogers, 1933; Piatt, 1940) has indirectly supported the contention of Lehmann and of Detwiler that mesodermal metamerism is primary. Very recent work by Humphrey (1947) on the human embryonic cord, however, has shown that stray sensory cells found within the central canal tend to clump together and adumbrate, to some extent at least, a type of segmental arrangement. No contact with somites is possible in Humphrey's cases and it is difficult to see what causal connexion could exist between the loci of these endomedullary cell groups and the mesoderm.

It would appear, finally, that whatever formative influence the mesoderm exerts over the segmental arrangement of the definitive cord resides in the axial mesoderm alone. Substitution of lateral plate mesoderm for brachial somites (Detwiler, 1937*b*) results in the formation of reduced and irregularly arranged spinal ganglia on the operated side. Forelimb rudiments (unsegmenting lateral plate mesoderm) grafted in place of brachial somites are not usually supplied with the normal number of nerves and the number of ganglia is reduced in the region of the transplant (Detwiler & Maclean, 1940). These latter results might be interpreted as some sort of mechanical interference with normal ganglion development but in the light of all the experimental data quoted it would seem unlikely; particularly, since scraping away of neural crest cells themselves (Detwiler, 1933*d*) does not result in abnormal spinal ganglion development.

It may be concluded that the axial mesoderm influences to some extent the metamerism of the central nervous system; precisely how far reaching and basic this influence is remains in doubt. It is commonly supposed that the splitting of the myotome itself into epimere and hypomere determines the further division of the spinal nerve into dorsal and ventral primary rami (Hamilton, Boyd & Mossman, 1945, p. 311). Observations of Sprague (1946) on the motor cell columns of the foetal sheep cord indicate that such an influence of the myotome is not actually a basic one, since he found no localization of motor cell groups relative to the distribution of the primary nerve rami.

III. DIFFERENTIAL GROWTH

Growth of the central nervous system as envisaged here includes those processes of proliferation and differentiation through which the increase, movements and structural specializations of the neuroblasts are brought about in the early embryo. The causal factors operating to fashion the morphological pattern and subsequent elaboration of form are sought. Before proceeding with a review of the more recent studies on this problem it is necessary to discuss briefly a few of the older theories yet extant.

(1) *Theories of Kappers and Bok*

The theories of Kappers (neurobiotaxis) and of Bok (stimulogenous fibrillation) are important because of the enormous amount of research and speculation they have engendered. These theories have had a tremendous heuristic value but it may be seriously questioned whether they have added much to our actual knowledge of neurogenetic processes and causal relationships in the early development of the central nervous system. Both theories are complementary and both have been based, for the most part, on non-experimental data. They are so highly speculative in nature that it is almost impossible to disprove them, and whenever a particular observation does not actually disagree with either theory one or the other is usually referred to (Pearson, 1946) in order to afford a theoretical explanation for the observations at hand. The only reason for mentioning them at all is their firm entrenchment in the literature of descriptive and experimental neuroembryology.

In a series of papers beginning in 1907 Kappers (1917, 1921, 1928) elaborated his theory of neurobiotaxis in order, chiefly, to explain the ontogenetic movements of various nuclei within the brain and the phylogenetic differences, or similarities, in position (Black, 1917) between homologous cell groups subserving different or altered functions. The so-called law of neurobiotaxis is formulated by Kappers as follows: 'If several stimulative charges occur in the nervous system, the outgrowth of the chief dendrites and eventually the shifting of the cells takes place in that direction whence the largest number of stimulations goes to the cell. This outgrowth or shifting, however, only takes place between stimulatively correlated centres; temporarily correlated excitation acts a part also in the connexions of the axons.' Neurobiotaxis leans heavily upon the presence of bio-electrical potentials and ionic polarization of the developing neuroblast to explain the growth of processes and the shifting of the cell body.

Kappers (1921) acclaimed the results of Ingvar (1920) as experimental proof of neurobiotaxis. Ingvar subjected tissue cultures of embryonic nervous tissue to weak galvanic currents. He observed that growth of the cell processes occurred almost entirely along the lines of force in the galvanic field. He noted also a morphological difference between those processes growing under cathodal or anodal influences, respectively. Ingvar's experiments have been repeated, with many modifications, by numerous workers but almost always with negative or indifferent results. Weiss (1933, 1934) after particularly thorough experimentation and analysis came to the conclusion that neither electrical nor chemical differentials have any direct effect upon the directional growth of nerve fibres. Marsh & Beams (1946), however, have recently claimed to have obtained directional nerve fibre growth under the influence of electrical currents. Regardless of what role electrical forces may play in the structural formation of the nervous system—and the positive evidence is very slight (Herrick, 1925)—the specific theory of neurobiotaxis as formulated by Kappers remains *sub judice*. The concept of neurobiotaxis by its very nature can have little value as an actual working hypothesis. It remains, in essence, simply a name for a particular type of observational data, not an explanation of these data.

The theory of stimulogenous fibrillation was formulated by Bok (1915*a*) following a purely descriptive study of the developing chick embryo. Most of his subsequent work (1915*b*, 1917) has been simply a theoretical elaboration based on his former data. Bok observed that the various cranial motor nuclei in the early chick central nervous system began their differentiation *pari passu* with the caudal progression of the fasciculus longitudinalis medialis. Postulated stimulation currents radiating from this tract were thought to cause the first formation of the motor axons away from the centre of stimulation and the growth of the chief dendrites, and later the cell body, toward this stimulation centre. He believed that other cell groups in the brain were stimulated to differentiate in a similar manner by appropriate tracts and fibre growth; among others he cites the position and differentiation of the oculomotor nucleus with respect to the optic pathways (1917). This theory is almost pure speculation and remains so to the present day. Also the language in which Bok couched his theory of stimulogenous fibrillation is so vague as to border on the mystical. For instance (1915*b*): '...the dendrite is rich in protoplasm, the neurite almost totally lacking in protoplasm. ...the nerve cell sends its dendrite out in the direction of its spiritual or functional nourishment'; and again, 'The stimulogenous fibrillation teaches us that the neurite chooses that plasmodesm which is the most frequently used.' Bok also invokes the Einstein hypothesis to account for certain neurogenetic phenomena.

Studies by Windle and his collaborators have more recently shown that even the observational data upon which Bok based his theory are wrong. Windle (1933), working on the neurofibrillar development in the embryonic brain of the cat, thinks that Bok's theory was based on an inadequate interpretation of facts. In the rat embryo (Windle & Baxter, 1936) it was observed that the first neurofibrils to develop are to be found in the motor nuclei of the cranial visceral nerves in the rhombencephalon, not the medial longitudinal fasciculus. In the chick embryo Windle & Austin (1936) observed that the sequence in development of cranial nerve nuclei is not that described by Bok but that the more posterior IX, X and XI develop first and VII begins its differentiation before VI.

It is not necessarily the purpose of this review to invalidate either neurobiotaxis or stimulogenous fibrillation. Certain of their points may some day prove to be correct. The importance of the discussion is to point out that when theories of forty or more years standing have received so little experimental substantiation it behoves us to invoke them less and to experiment more.

(2) *Cellular proliferation and differentiation*

(*a*) *Intramedullary factors*

Factors thought to control the early differentiation and growth of the neural axis as a whole have been discussed. Those factors responsible for either the proliferation or differentiation of the individual neuroblasts and their *inter se* relationships are exceedingly obscure. Holtfreter (1931*a*) showed that when presumptive neural tissue from early amphibian gastrulae was implanted into the coelomic cavity it gave rise, among other things, to large neurons which he thought to be Mauthner's cells. Raven (1935) obtained cells resembling mesencephalic V root cells from ectopic grafts of presumptive cranial fold tissue taken from late gastrulae. Grafted pieces of neural plate or neural fold tissue from stage 15 in *Amblystoma* were also found to give rise to morphological Mauthner's or mesencephalic V root cells respectively (Piatt, 1945). Although the identification of Mauthner's cells in some of these cases was questionable, there was little doubt as to the proper allocation of the mesencephalic V root cells. Windle & Fitzgerald (1942) concluded that in human embryos the mesencephalic V nucleus differentiates in a caudo-cephalad direction and that some of these cells might actually be derived from migratory trigeminal motor neuroblasts. In Amphibia the greater part of these cells would seem to arise *in situ* from neural fold material (Piatt, 1945). These results indicate that as early as the gastrula or early neurula stage in amphibians the determination of specific types of nerve cells is already on its way. Determination of this sort cannot be considered strictly completed at the time of transplantation, since the definitive neurons in question were never entirely isolated from surrounding nervous tissue. Experiments (Detwiler, 1927*d*, 1933*b*) have indicated that, following its extirpation, Mauthner's neuroblast is often replaced as late as stages 27–29 in *Amblystoma*. The work of Hoadley (1924, 1925*a*) has shown, in addition, that pieces of the early chick mesencephalon and cord undergo a remarkably complete differentiation when isolated on the chorio-allantoic membrane. He did not mention mesencephalic V root cells. It would have been interesting to know if these most characteristic of all mesencephalic cells differentiated in the isolated pieces. Other experiments on chick embryos (Danchakoff & Agassiz, 1924; Rudnick, 1938) indicate that isolated parts of the medullary plate are able to undergo considerable self-differentiation. Experiments of this nature give some evidence that cells and specific parts of the central nervous system are capable of undergoing a very early self-differentiation but they do not elucidate the neurogenetic forces which bring this about.

It has been repeatedly pointed out by Coghill (1924*a*, 1929), and others, that the differentiation of a neuron continues even after function has commenced and that true differentiation does not imply an all-or-none type of trigger mechanism. Differentiation continues for a relatively long period and during that time the neuron responds to its own inherent capacities (Coghill, 1930), as well as to its environment. Coghill (1924*b*, 1926*a*, 1928) has shown by his meticulous study of the developing amphibian nervous system, that proliferation is not only different from differentiation but that for a given region the ascendency of the one tends to prevent the acceleration of the other. Coghill (1933*a*) showed that, although mitotic counts in successive spinal segments gave an exceedingly choppy graph, a graph representing the total number of cells per segment falls gradually from anterior to posterior. This, and additional work along the same line (Coghill, 1933*c*, 1936), indicated to Coghill that localized differences in proliferation rates are compensated by a dynamic regulation of the whole and that bilateral asymmetry tends to decrease progressively with age. The process of differentiation (Coghill, 1926*a*) is likewise under the control of factors inherent within the nervous system, the broad features of which are anticipated as early in development as the medullary plate stage. He believed, in general, that the capacity of the central nervous system to increase and differentiate is not dependent upon the extra-nervous environment but resides within the dynamic equilibria of the nervous tissue itself. The majority of Coghill's work is predominantly descriptive and analytical in nature and is concerned chiefly with the field of functional neurogenesis. His research, however, has served to clarify several principles of purely structural neurogenesis, particularly the distinction between mere increase in cell number (proliferation) and the specialized growth of the neuroblast itself (differentiation).

A long series of papers, chiefly by Detwiler and his associates, has thrown some light on the factors which influence cellular proliferation in the amphibian spinal cord. Detwiler (1920*b*, 1923*a*) replaced brachial segments 3–5 by smaller, more posterior segments 7–9. Neuroblasts in the transplanted segments underwent an increased proliferation approximately equal to that which characterizes the normal brachial development. When segments 1–3 were substituted for segments 4–6 (Detwiler, 1925*c*) the dorsal sensory region of both intact host and grafted segments 1–3 underwent a marked increase in size and cell number. The ventral motor regions, on the other hand, showed a decrease in both size and cell number. Finally, replacement of segments 2–4 by segments 4–6 (Detwiler, 1929*b*) resulted in a marked cellular hyperplasia of transplanted segments 5–6 but only a negligible 6% increase in segment 4. Detwiler concluded from these studies that spinal cord segments 1–2 possess an inherent capacity for proliferation while more caudal segments adjust themselves to their position in the antero-posterior gradient and thus show less inherent ability to maintain their normal mitotic rate and size. This conclusion does not satisfactorily explain the failure of the fourth segment to increase when occupying the position of the host second. If position is all important below segment 2, why did not the fourth undergo a greater hyperplasia than transplanted segments 5 or 6? Detwiler explains the difference in response between sensory and motor areas of segments 1–3, following transplantation to a more posterior level, as due to a greater prolongation of inherent capacities for self-development in the dorsal (sensory) part of the embryonic neural axis. Cell proliferation is considered to be regulated to a great extent by longitudinal fibre tracts growing into the various cord segments. Experiments by Maclean (1932) and Moyer (1943) substantiate, for the most part, the data and conclusions of Detwiler.

Experiments in which certain segments of the embryonic *Amblystoma* cord were reversed end for end (Detwiler, 1923*b*, 1928) give added proof that more anterior cord levels possess a greater inherent capacity for self-development than do more caudal ones. When segments 1–3 are reversed, segment 1 in its more caudal position manifests a greater cell count than does segment 3 which is now situated more anteriorly. Cell counts in reversed segments 3–5, on the other hand, show an approximately normal anterior > posterior gradient. When segments 1–3 are reversed end for end and substituted for segments 3–5 (Detwiler, 1930*b*), they behave differently, however, than when they are reversed in the orthotopic position. Although the entire grafted unit maintains a greater cell count than the displaced segments 3–5, the respective cellular proliferation of the individual reversed segments falls into line with an anterior > posterior gradient. In none of these experiments was the trauma of operative procedure itself responsible for the altered cell counts (Detwiler, 1925*b*; Detwiler & Maclean, 1932). Detwiler concluded that the most anterior spinal cord segments behave as a fixed developmental unit but that the individual segments may undergo morphogenetic alterations depending upon their position within the whole. Such a view is probably more correct than the idea of a rigid, non-plastic development for certain rather arbitrary segments of the spinal cord and morphogenetic plasticity in others. After all, the spinal cord develops pretty much as a structural unit; its early formation shows little evidence of true metamerism. A gradually diminishing *A–P* structural gradient would be expected, but hardly a stair-step type of delimitation.

Heteroplastic grafts of spinal cord units between *Amblystoma punctatum* and *A. tigrinum* were first made by Wieman (1925*b*, 1926), but demonstrated little more than that such grafts were possible and formed an anatomical and functional part of the whole. Detwiler (1931, 1932*b*, *c*) has extended Wieman's earlier work. He found that when *punctatum* brachial segments were replaced by those of *tigrinum* the grafted unit eventually regulated so as to correspond in size and cell number with a normal *punctatum* brachial region. During the first 3 weeks, or earlier, the *tigrinum* graft exceeds both normal donor and host cords in its development. When *tigrinum* segments 1–3 are substituted for *punctatum* 3–5, the grafted *tigrinum* unit does not completely regulate itself to conform to its new position in the *punctatum* cord. These results are like the preceding in demonstrating a relative independence of growth for the most anterior cord segments and a greater influence of position on the more caudal ones; also, that factors of regulability transcend the species level.

The experiments already discussed have demonstrated, in general, that cellular proliferation within a given part of the spinal cord is controlled to a large extent by relative anterior-posterior locus, cell number decreasing more or less gradually from anterior to posterior. The proximity of segments 1–2 to the longitudinal projection tracts (bulbo-spinal and tecto-spinal), issuing from the caudal end of the medulla, tended to indicate that descending growth of these fibres might be responsible for the increase in cell number obtained in the more anterior position. To test this supposition Detwiler (1924*c*, 1925*a*) grafted an extraneous medulla in place of cord segments 1–5. He found that the segments immediately caudal to the grafted medulla increased in size, volume and cell number, as compared to those same segments (6–7) in normal controls. The transplanted medulla in these and other experiments (Detwiler, 1927*c*) suffered a cellular hypoplasia. Detwiler concluded that the hyperplasia of host segments 6–7 was due to the additional projection fibres growing caudally from combined donor and host medullae. No attempt was made to ascertain if the number of projection fibres was actually increased at the level of host segments 6–7. It is possible that those from the host medulla may have failed to traverse the entire length of the graft. Experiments by Nicholas, also on *Amblystoma*, tend to confirm the results of Detwiler. When an extra medulla was added (Nicholas, 1931), a hyperplasia of cells occurred in the ventral motor area of those segments of the cord lying posterior to the graft. By blocking the central nervous system with implants of non-nervous tissue Nicholas isolated the anterior part of the cord from either medulla (1929) or forebrain (1930). He found that in the former instance brachial segments (3–5) showed a cellular hypoplasia of 40%; in the latter experiment no hypoplasia was observed. These results led Nicholas to believe that the descending fibres from the medulla were the dominant agent in effecting cellular proliferation within the spinal cord. Experiments on chick embryos (Williams, 1931) also indicate that descending and ascending fibre tracts help to regulate the number of cells in the lumbar region of the cord.

The role of longitudinal fibre tracts in stimulating cell proliferation within the spinal cord is actually a doubtful one. Severinghaus (1930) obtained tremendous hyperplasias in isolated, heterotopic cord grafts and concluded that fibre tracts could not have been operative in such cases. Zacharias (1938) later showed that the hyperplasias recorded by Severinghaus were not actually true when taken on a segmental basis; however, in her heterotopic cord grafts she demonstrated that real hyperplasias can occur if the operation is performed early enough. Detwiler (1933*c*) also showed that cellular proliferation is not necessarily decreased in isolated cord grafts. Furthermore, when spinal cord is substituted for medulla (Detwiler, 1937*a*) cellular proliferation caudal to the graft proceeds, in most cases, just as well in the absence of bulbo-spinal tracts. On the basis of such experiments Detwiler himself has questioned the conclusions of some of his earlier work. Levi-Montalcini (1945) removed a relatively large unit of the cervical spinal cord in 38 hr. chick embryos, thus preventing the down-growth of spinal projection tracts into lower cord levels. She found no decrease in volume of the thoracic and lumbar grey substance, and motor cell counts were equal in operated and control specimens. Hamburger (1946*a*, *b*), by an ingenious use of tantalum foil, isolated *in situ* the brachial segments in the chick embryo from both descending and ascending tracts. The amount of both grey and white matter in these isolated segments was actually slightly more than in the controls. Motor cell counts were practically the same in blocked and control cords. Hamburger concludes that both proliferation and differentiation proceed normally in the absence of longitudinal tracts. These more recent experiments, coupled with others to be mentioned below, strongly indicate that cellular proliferation within the central nervous system is not controlled by either presence, kind or amount of invading fibre tracts.

It has been pointed out that cellular increase is not the same process as differentiation. Evidence from various sources (Faris, 1924; Hinrichs, 1927; DuShane & Hutchinson, 1941; Gillette & Bodenstein, 1946) clearly demonstrates the dissociability between the two processes. Despite a few observations to the contrary (Hamilton, 1901) it is now generally recognized that, once a normal neuroblast reaches an advanced stage in its development, it no longer divides. It has even been suggested (Craigie, 1924) that the earlier phases of neural differentiation may be different from the later functional stages, although this is largely a matter of definition and requires further evidence. What, then, are the processes at work within the nervous tissue itself which might serve to initiate or control the purely differentiation phase of growth?

Coghill (1931) showed that in *Amblystoma* cerebral differentiation begins simultaneously in several isolated primordial regions and he could find no evidence that growth of nerve fibres into a region activates the differentiation of neuroblasts or influences their polarity. Szepsenwol (1935) thought that Mauthner's cell differentiates in the axolotl under the influence of motor impulses. Herrick (1937) is of the opinion that local differentiation is not initiated by any extrinsic agent, although he believes (Herrick, 1922) that local proliferations may be initiated by the growth of definite fibre tracts into an area. More recent observations by Barron, however, would indicate that invasion of a territory, containing undifferentiated, indifferent neuroblasts, by growing nerve processes does in fact cause the beginning of differentiation in these cells. In the formation of the motor columns of the sheep spinal cord Barron (1943) noticed that indifferent cells begin to send forth their processes only at the time that dendrites of other cell columns invade their territory. Sensory cells of the spinal ganglia also began to differentiate and give rise to processes only after ventral root fibres made contact with the ganglia, and further growth of the central process of these early differentiating ganglion cells seemed in turn to stimulate other indifferent cells to differentiate (Barron, 1944). This close association in time and space between the first visible differentiation of cells and the invasion of the region by growing dendrites was also observed to occur in the spinal cord of the chick (Barron, 1946). These studies led Barron to conclude that growing dendrites which invade an indifferent field have the capacity to induce these indifferent cells to differentiate into neuroblasts. Barron's studies are important since they furnish about the only evidence we have that differentiation is dependent upon the proximity of developing nerve fibres. They are not critical, however, in proving that such a space-time event is actually one of cause and effect. Furthermore, the theory can offer no explanation for those cases of early, abortive and transitory differentiations reputed to occur in other forms (Angulo y González, 1939, 1940; Humphrey, 1944; Youngstrom, 1944). One would like to know also why secondary motor cells in the amphibian spinal cord (Youngstrom, 1938, 1940) remain in the undifferentiated state long after many neurofibrils are found about their cell bodies. Granting that fibre invasion causes indifferent cells to differentiate, it seems probable that such a stimulus is a highly specific and selective one. The causal relationships effecting neural differentiation are still very obscure.

(*b*) *Extramedullary factors*

It has been recognized for some time that extramedullary factors and peripheral fields exert considerable influence upon the morphogeny of the central nervous system. The pioneer work of Steinitz (1906), Braus (1906), Shorey (1909) and Dürken (1911, 1913) showed clearly that ablation of peripheral areas results in a marked reduction and hypoplasia of the corresponding nerve centres. The experiments of Kopeć (1922), Ranzi (1928) and Power (1943, 1946) have demonstrated that this general principle is not limited to the vertebrate nervous system alone but appears to operate effectively in insects and other invertebrates. The mechanism by which peripheral control is implemented, however, is poorly understood. Shorey (1911) thought that the development of motor neurons depended upon the catabolic products of muscular activity. There has been no substantiation of her contention.

As pointed out by Harrison (1935), the effect in ablation of primary sensory neurons (nose, eye, ear) is relatively easy to comprehend, since the number of nerve fibres entering the brain would be reduced. In the case of ganglion or motor cell hypoplasia the reason is less clear, for the removal of the peripheral organ (skin, muscle) does not directly affect those cells and their processes which are present. In the latter case Hamburger (1939*a*) assumes an intranervous mechanism rather than a growth-stimulating substance travelling in a centripetal direction.

De Burlet & Ströer (1940) have pointed out that it is the actual presence or absence of a sense organ and not function which affects the growth and development of brain and ganglia. Experimental evidence indicates that such is probably the case. Excision of the nasal placode in *Amblystoma* (Burr, 1916*a*, *b*) resulted in a reduced development of the ipsilateral cerebral hemisphere, which effect was later shown (Burr, 1920) to be independent of nasal function. Ingrowth of fibres into the brain from supernumerary olfactory organs (Burr, 1923*a*) or from larger *tigrinum* olfactory organs in *punctatum* hosts (Burr, 1930) causes a cellular hyperplasia of the corresponding hemisphere. Weissfeiler (1924) demonstrated that a regenerating olfactory nerve in adult urodeles excites cellular proliferation, and probably differentiation, at the region of entrance into the brain. With regard to the eye Larsell (1929, 1931) showed that unilateral excision of this organ in larvae of *Hyla* caused a cellular hypoplasia of the contralateral optic tectum. He regarded this effect as due to lack of functional stimuli entering the optic centres. Experiments by Goodman (1932), however, have demonstrated that lack of functional stimuli reaching the optic centres have absolutely no effect upon the development of the brain. Rabbits with enucleated eyes were born and raised with normal controls in utter darkness for a period of 6 months. The eyeless rabbits showed developmental retardation of the superior colliculus and lateral geniculate body connected with the side of operation; the rabbits with eyes present possessed completely normal midbrain structures. Doubled or supernumerary eyes (Pasquini, 1927) in *Pleurodeles* cause a cellular hyperplasia of the brain proportional to the number of entering fibres. Heteroplastic grafting of eyes between *Amblystoma punctatum* and *A. tigrinum* (Harrison, 1929; Twitty, 1932) has shown that midbrain hypoplasia or hyperplasia is directly related to the number of entering optic fibres, the *tigrinum* optic nerve having many more fibres than that of *punctatum*. If a particular peripheral field is removed to make room for a transplanted eye, the corresponding part of the central nervous system shows a hypoplasia if the ectopic optic fibres make no central connexions (Nussmann, 1931). Eyes grafted in place of the ear in *Amblystoma* (May & Detwiler, 1925) cause marked hyperplasias in both IX–Xth ganglion complex and medulla when the optic fibres make connexion with these structures. This proliferation is not due to any injury occasioned by the operation (Detwiler, 1927*b*) and demonstrates the non-specific nature of the end-organ stimulus. On the other hand, the absence of the ear in these experiments resulted in a very marked hypoplasia of the VII–VIIIth ganglion complex. However, such a result can hardly be considered a hypoplasia in the ordinary meaning of the term since undoubtedly most, if not all, of the 60% 'hypoplasia' recorded was due to removal of the VIIIth ganglion primordium along with the ear. Experiments by Richardson (1932), though, have demonstrated beyond doubt that VIIIth nerve fibres do affect the cellular proliferation of both the acoustico-geniculate ganglion complex and area acoustica of the medulla in *Amblystoma*. None of these studies sheds much light on the dynamic mechanism whereby ingrowth of primary sensory nerve fibres regulates the amount of cellular proliferation and differentiation within the central nervous system, but they are conclusive in demonstrating that such a regulation does occur.

The extent to which the non-nervous periphery influences the development of nerve centres has become clear only in recent years. Detwiler and his associates (Detwiler, 1920*a*, 1921, 1924*a*, *b*; Detwiler & Carpenter, 1929) first showed that in urodeles removal of a forelimb causes extensive cellular hypoplasia of the brachial ganglia and that heterotopic limbs increase the number of cells in the associated ganglia. These results have been substantiated by Balinsky (1927), Wieman & Nussmann (1929) and Schwind (1931) for the forelimb, and by Lovell (1931) for the hindlimb. Carpenter has demonstrated a hyperplasia of spinal ganglia associated with heterotopic limbs grafted in both larval stages (1932) and after metamorphosis (1933) in urodeles. Heteroplastic grafting of larger *Amblystoma tigrinum* limbs to *A. punctatum* hosts also caused a hyperplasia of either spinal (Detwiler, 1930*c*) or cranial (Detwiler, 1930*a*) ganglia, depending upon the locality of the graft. Hyperplasias obtained from these heteroplastic grafts were relatively slight considering the much greater size of the *tigrinum* grafts; and *tigrinum* somites substituted for those of *punctatum* (Detwiler, 1938) showed hardly any hyperplasia of associated ganglion cells. Detwiler attributed these unexpectedly low hyperplasias to the fact that *tigrinum* musculature owes its much greater bulk primarily to increase in muscle fibre size rather than number. Harrison (1924) had previously reported no hyperplasia following heteroplastic grafts but later modified his earlier conclusions (Schwind, 1931). Carpenter & Carpenter (1932) reported hyperplasia of ganglia following transplantation of larval limbs, but found no correlation between amount of muscle volume and degree of cellular increase. Studies by Detwiler (1926*a*, 1927*a*), however, indicate that the hypoplasia following reduction of the peripheral field is due more to skin loss (60%) than to muscle loss (40%). A few workers (Nicholas, 1924; Weiss, 1928, 1937) have been unable to discover spinal ganglion or motor cell hyperplasias following limb grafting in urodeles, but it is generally agreed that the results of the above experiments are, for the most part, well substantiated.

Although ganglion hypo-hyperplasia has been shown to result from volume alterations in the non-nervous peripheral field in urodeles, Detwiler (1926*b*, 1933*a*, 1936, 1940*a*) has steadfastly maintained that the number of primary motor neurons is unaffected by changes at the periphery. Detwiler & Lewis (1925) recorded a slight reduction in the size of motor neurons following limb excision and an appreciable reduction in number of motor root fibres, but found no deviation from the normal cell number. Recent experiments by Stultz (1942), however, have demonstrated conclusively that motor cell hypoplasias exceeding 50% may result from ablation of peripheral fields in *Amblystoma* if the counts are made after metamorphosis. He concluded that the acquisition of these responses is a progressive and gradual one, i.e. the older the animal the more marked the hypoplasia. Stultz also recorded motor cell hyperplasias. Experiments by Piatt (1946) have shown that the number of intramedullary sensory cells of the first order (mesencephalic V root nucleus) in *Amblystoma* is likewise influenced by the non-nervous periphery. From the results of these last two studies it would appear that there is no essential difference between the response of intramedullary nerve cells and cranio-spinal ganglion cells to volume changes affected on their respective end-organs. Detwiler's contention that the extent of intramedullary neuron proliferation is determined wholly by intracentral forces is no longer tenable.

In anurans May (1930*a*, *b*, 1932, 1933) has shown clearly that both spinal ganglion and motor cells of the lumbosacral region of the spinal cord are dependent upon the presence of the hindlimb for their normal development. If innervation of the hindlimb is prevented, a marked reduction of motor neurons and spinal ganglion cells follows. This is also true for the anuran brachial region (May, 1937). The spinal cord of a frog found in nature possessing three functional hindlimbs on its right side has been described by Bueker (1945*b*). He found a large ganglion hyperplasia on the right side but the total cell count of the right ventral portion of the lumbosacral region of the cord showed no increase over that of the normal left side. There was, however, a decided increase in the cell count of the lateral motor column on the right. Weiss (1931) has also recorded observations on a frog found with three left forelimbs. He considered the slight increase of dorsal root fibres present on the abnormal side of no significance. No motor cell counts were made but the brachial region of the cord was symmetrical. The predominant evidence indicates that both motor and sensory cells are affected by peripheral changes in anurans.

In birds the results compare favourably with the data obtained in anuran experiments. Hamburger (1934) showed that in the chick wing bud extirpation results in an average spinal ganglion hypoplasia of 45%, and for lateral column motor cells 22–61%. When additional wings were transplanted, the lateral motor cell count was increased 21–27%; when additional hindlimbs were transplanted, the lateral motor column of the lumbosacral region showed cellular hyperplasias as high as 88% although the average was considerably lower than this (Hamburger, 1939*b*). Spinal ganglion hyperplasia also occurred in both instances. Baumann & Landauer (1943) made a study of the adult motor columns in the lumbosacral region of a polydactylous strain of chicks and found that those columns supplying the more distal limb segments (dorsolateral and retrodorsolateral) showed a consistent cellular hyperplasia. The highly important series of experiments by Bueker (1943, 1944, 1945*a*) has completely substantiated Hamburger's observations. Bueker's work is too complicated and varied to mention in detail. His general results show: (1) extensive hypoplasias of lateral motor (75–90%) and ganglion (50%) cells following limb ablation, (2) response not species specific, (3) almost complete unimportance of intracentral factors (longitudinal tracts) in controlling number of motor neurons.

Tissue culture experiments by Szepsenwol & Goldstein (1938) and Szepsenwol (1940*b*, *c*) give added proof that, in the chick, the periphery (somites) exerts an influence over neuron differentiation and fibre growth. Szepsenwol (1940*a*), however, is of the opinion that longitudinal projection fibres are important and necessary for initiating normal neuron differentiation.

In reptiles only one paper comes to light in the present connexion. Terni (1920) amputated the tail in a species of lizard and found, after regeneration had occurred, that the new tail was innervated entirely by nerves arising cephalad to the regenerate. This new condition increased the peripheral field of these nerve centres and Terni reports cellular hyperplasias in these end ganglia as high as three times that of the normal. This work shows that the nervous system of reptiles (a group offering as yet little experimental data) falls into line with other vertebrate groups.

In mammals the importance of the end-organ on the development of the central nervous system is also well established. Significant reduction of spinal cord motor cells has been recorded in a case of human abrachia (Curtis & Helmholz, 1911) and congenital absence of a part of the hindlimb in a rabbit (Romanes, 1942). Hyperplasia of motor cells was observed by Tsang (1939) in polydactylous mice, although it was interpreted by him as cause rather than effect. Amputation of forelimbs in foetal rats (Hall & Schneiderhan, 1945) and sheep (Barron & Barcroft, 1938; Barron, 1945*a*) has resulted in both ganglion and motor cell hypoplasias of the associated nerve centres.

The regulative influence of the periphery on both ganglion and intramedullary neurons has been well established in all major vertebrate groups studied. Positive evidence has been presented above (Stultz, 1942; Piatt, 1946) that the intramedullary cells of urodeles can no longer be considered an exception to this general principle. An extremely important paper by Hamburger & Keefe (1944) has indicated the reason for the former difference of opinion concerning the response of urodele intramedullary cells. They found that wing bud ablation in the chick caused a hypoplasia of differentiated motor neurons but no reduction in the total number of cells composing the ventral quadrant of the ipsilateral brachial cord. Total cell counts were equal on operated and unoperated sides; only the purely motor cells were reduced in number. Also, counts of dividing cells on both operated and unoperated sides during the peak of mitotic activity were the same. They point out, further, that all of Detwiler's 'motor' cell counts were actually total cell counts of the entire ventral half of the cord; in larval urodeles it is very difficult to distinguish motor cells from intercalated and commissural cells. Therefore they conclude that Detwiler's and their data do in fact agree. Total cell counts in both urodele and chick are not affected by alteration of peripheral tissue; true motor cells in the chick are affected, and presumably, if they were counted separately in the urodele (as done by Stultz), this latter group would also show a motor cell response. On the basis of this analysis they postulated that 'the deficit of motor neurons in hypoplasia was balanced by an excess of non-motor neurons, and the surplus, in the case of motor hyperplasia, was compensated for by a deficit in non-motor cells.' In this connexion it is of interest to note that Perry (1942), following unilateral forelimb excision in *Amblystoma*, recorded a greater number of dorsal and ventral commissural neurons on the operated side than on the normal. Perry counted all non-motor cells as commissural cells and Hamburger and Keefe point out that some of Perry's 'commissural' cells were very likely undifferentiated motor cells. The work of Hamburger & Keefe (1944) and Stultz (1942) gives us good reason to believe that in urodeles true motor areas undergo cellular hypo-hyperplasia, following peripheral alteration. There is at present no foundation for believing that first order, intramedullary neurons are wholly under the influence of intracentral factors in urodeles (Piatt, 1946).

Most recent investigators consider peripheral regulation of specific nerve centres to be largely a matter of differentiation, not proliferation. Hatai (1902), and others, showed some time ago that mitotic activity within the central nervous system occurs chiefly in the early stages, and the experiments of Hamburger & Keefe (1944) and Barron (1945*a*) indicate that simple increase or decrease of undifferentiated neuroblasts is probably not a factor. Studies by Romanes on the cell columns of the human (1941*a*) and rabbit (1941*b*) spinal cords offer indirect evidence that the limbs influence the organization of the central nervous system, a type of relationship more akin to differentiation than to cell proliferation. Support for the control of the periphery on differentiation comes from recent experiments (Piatt, 1947) on the differentiation of Mauthner's cell in *Amblystoma*. When the primordia of ear and VIIIth root fibres were removed, Mauthner's cell failed to differentiate in about 33 % of the cases. Experiments by Greene (1947) substantiate these data to some extent. When the VIIIth nerve was made to enter the brain at abnormal loci, a Mauthner's cell occasionally developed at or near the point of nerve entrance. These results were interpreted as evidence for the influence of ear and VIIIth nerve on the differentiation of Mauthner's cell. The important point in this connexion is that, since only one Mauthner's cell is normally present on each side of the medulla, its presence, or lack of it, cannot be a matter of cell proliferation. Failure of a single cell of a specific type to develop can only mean it has not differentiated; to speak of lack of proliferation into one cell is nonsense.

The actual mechanism whereby the periphery regulates the differentiation of neuroblasts is not known. Barron (1943, 1945*a*, 1947) suggested that contact with peripheral elements by the distal process of a nerve cell causes the dendrite of that cell to start development. The dendritic process, in its turn, initiates the differentiation of neighbouring indifferent cells, and this process is repeated until the periphery becomes saturated with nerve fibres. When this point is reached, no more stimulus is present for dendritic growth and hence no further differentiation of indifferent cells occurs. Hamburger & Keefe (1944) and Hall & Schneiderhan (1945) accept this tentative hypothesis. Experimental analysis by Levi-Montalcini & Levi (1942) suggests, however, a slightly different explanation. They found that after hindlimb extirpation in chick embryos the number of undifferentiated ganglion cells on both control and operated sides were equal. This equal ratio was maintained up to the time when mitotic activity normally ceases. The number of differentiated cells, on the other hand, became progressively less as older and older animals were examined. From these data they postulate that hypoplasia is accomplished by an increasingly widespread atrophy of already differentiated cells which have failed to make their normal synaptic connexions. Barron (1944) also suggested that the periphery may not regulate cell number by an initial differentiation stimulus (modification of his previous theory) but controls number by the fact that only those cells survive whose distal processes have made functional contact. He also pointed out (1945*b*) that regulation of motor neuron number may be implemented by a different mechanism than that operative in the case of spinal ganglia.

Other extrinsic agents, such as hormones (Hoskins & Hoskins, 1919; Cooksey, 1922; Allen, 1924; Hammett, 1926), undoubtedly affect the structural development of the central nervous system. Recent chimera experiments made on amphibian embryos suggest the importance of non-nervous tissue specificity (Rath, 1945) in normal spinal cord development, rather than inherent conflict between species in different parts of the neural axis itself. Position in the *A–P* neural axis, rather than tissue specificity of the adjacent non-nervous tissue, may also be a factor in susceptibility to altered conditions (Hadorn, 1945). It is no longer possible to minimize, as so often has been done in the past (Lane, 1917), the influence of extra-neural structures and forces on the development of the neural axis.

(3) *Fibre pattern*

The causal factors which determine the eventual fibre patterns within the central nervous system are not known. Herrick (1933) stated several years ago that the mechanics of the directive influence which induces growth of axons into any specific field is as yet an unsolved problem. He (1930) also drew attention to the fact that in the lower vertebrate brain, at least, preferential functional pathways are so imbedded in dense neuropil tissue that no clear cut, structurally isolated system of fibre paths can be said to exist, in the diagrammatic manner in which they are usually conceived. Certain experiments by Weiss (1940, 1941*b*), in which isolated, mashed or otherwise deranged fragments of spinal cord were made to innervate ectopic limbs in *Amblystoma* larvae, show that reflex movement is not dependent upon the finer structural differentiation found in normal intact spinal cord. Weiss (1941*c*) concluded that all nerve cells, regardless of position, tend to discharge spontaneously but are in some way repressed by the structural organization of the nerve centres. The primary motor patterns (Weiss, 1941*d*) arise essentially by self-differentiation within the central nervous system independently of the influence of sensory control. Experiments by Harrison (1904) and later by Matthews & Detwiler (1926) have shown that amphibian embryos raised in chloretone perform co-ordinated, normal movements almost immediately following removal from the anaesthesia. The differentiation of normal structural patterns within the central nervous system is shown by such experiments to be independent of function. Recent experiments by Sperry (1946) give evidence that training plays no part in the proper establishment of vestibulo-ocular association pathways but that the pattern is laid down in a predetermined manner. The purpose of citing these references and conclusions here is simply to point out that the purely structural organization and normal growth of intramedullary fibre systems are probably unrelated to the functional aspect of development.

Some experimental evidence exists for directional stimuli of fibre growth within the central nervous system. Hooker (1917) reversed end for end a section of the anterior part of the spinal cord in neurula stages of *Rana*. He noticed a marked tendency for nerve fibres to avoid entering the opposite ends of the wound surface of the cord. When a unit of embryonic cord was rotated 90 and 135° on its longitudinal axis so that dorsal-ventral relationship was deranged (Hooker, 1922, 1930), the fasciculi exhibited torsion in a regular fashion to gain the corresponding fasciculi in the rotated segment. Following 180° rotation, dorsal and ventral developing fibre tracts often formed dorso-ventral decussations to gain like regions of the graft. Hooker interpreted these results as strong evidence for directional growth of longitudinal tracts and specific attraction stimuli in the developing central nervous system. Wieman (1922) rotated anterior units of embryonic *Amblystoma* cords 90° around the dorso-ventral axis. The descending fibres growing from the anterior wound surface of the cord entered the rotated piece near its originally anterior end, and later ascending fibres tended to enter the originally posterior end of the graft. When 90° rotation was performed on more posterior cord segments, mechanical blocks arose between intact cord and rotated piece before descending fibres could reach the latter. Pieces rotated 135° also failed to become connected by growing fibres with remainder of the cord (Wieman, 1925*a*). Wieman thought his data showed the existence of specific attraction forces in the polarity of growing fibres and a growth repulsion when original polarities were too greatly disturbed, i.e. 135° rotation. In anurans May (1927) transplanted pieces of telencephalon together with either eye or olfactory organ. Nerve fibres from both eye and nasal sac grew into the transplanted brain pieces. May considered this a form of neurotropism but of a non-specific nature. Hoadley (1925*b*) could find no evidence of specific attraction forces operating in the nerve fibre growth of chick mesencephalon grafted to the chorio-allantoic membrane. None of these studies has indicated the true nature of growth polarities or attraction stimuli thought to influence fibre pattern growth within the central nervous system.

Mechanical contact with solid substrata or other fibres has long been thought to be an important factor in guiding the developing nerve fibres (Harrison, 1912, 1914). Descriptive studies by Tello on the developmental sequence of fibre tract origin in chick (1923) and mouse (1934) embryos, by Rhines & Windle (1941) on rat, cat and man and experiments by Rhines (1943) on the chick have shown that the great longitudinal fibre tracts are the first to develop. Chief among these is the medial longitudinal fasciculus which Tello thought might serve to guide other tracts appearing somewhat later. On the basis of these general facts Oppenheimer (1941) performed induction experiments on *Fundulus* eggs. The accessory brain structures produced gave rise to supernumerary Mauthner's cells in midbrain and medulla.

Most of the fibres from these ectopic cells joined the primary neural axis and followed their normal routes posteriorly in close conjunction with the medial longitudinal fasciculus. Oppenheimer concluded that purely mechanical forces were unimportant in directing the supernumerary Mauthner's fibres but that the medial longitudinal fasciculus exerted some sort of attraction on the descending axons and served to guide them to their normal destination. Experimental data on supernumerary Mauthner's cells in *Amblystoma* (Piatt, 1943), on the other hand, give no evidence that ectopic Mauthner's fibres are directed in their course by the medial longitudinal fasciculus, nor that they are associated with any other specific tract for any distance. The fibres showed remarkable independence in their growth; a few grew outside the central nervous tissue for varying distances. In one instance a posteriorly growing fibre turned abruptly at the level of the third spinal nerve and retraced an anterior route as far as the mesencephalon. Mauthner's axon decussates with its fellow of the opposite side near its place of origin from the cell body and follows the contralateral fasciculus longitudinalis medialis posteriorly in the spinal cord. Based on further experimental data, Oppenheimer (1942) postulated that the reason for decussation and contralateral posterior growth of Mauthner's axon was a space-time relationship between it and the growth of the medial longitudinal fasciculi. The axon crosses the midline before the ipsilateral fasciculus has reached this level and turns posteriorly to follow the descent of the contralateral fasciculus because the latter has by this time attained the level of the axon. Oppenheimer's hypothesis of decussation was re-examined in experiments on *Amblystoma* with negative results (Piatt, 1944). It was concluded that the fasciculus longitudinalis medialis has no necessary effect on either the decussation or posterior growth of Mauthner's axon in the form studied. Such a conclusion appears all the more likely since Coghill (1926*b*) stated that he could not yet identify Mauthner's fibre in the early swimming stage, although by this stage of development the fasciculus longitudinalis medialis has passed beyond this level of the medulla. Rhines (1944) and Rhines & Windle (1944) performed various types of ablation experiments on embryonic chick brains and found that ventral commissures and fibre decussations were still present in the absence of ventral longitudinal tracts. They could discover no relation between the presence of longitudinal tracts and the course taken by fibres after decussation. They concluded that directional fibre growth in the chick is influenced chiefly by substrate configurations and denied the evidence of any trophic influence exerted by adjacent nerve centres.

Recent experiments by Sperry (1944, 1945*b*, 1947) on larvae and adults of amphibians, although not directly related to the problem of structural neurogenesis, give strong evidence that centripetally regenerating fibres of various cranial nerves establish an orderly and functional connexion with the proper brain centres in certain cases. Sperry (1945*a*) was led to conclude that the same predetermined growth-regulating factors responsible for the development of anatomical and physiological neuron relationships in ontogeny were also operative in adult nerve regeneration in amphibians. The selective growth of regenerating fibres toward specific nerve centres in post-embryonic brains indicates either point for point anatomical growth or unusual plasticity in physiological regulability. It is not possible to choose between these alternatives at this time.

(4) *Superficial origin of cranio-spinal nerves*

Relatively few studies are germane to the problem of cranial and spinal nerve origin from the central nervous system. Lewis (1906*b*) long ago noted that localized injuries to the embryonic brain of *Amblystoma* constitute points of exit for aberrant nerves which may develop, and that nerve fibres from ectopic optic vesicles will enter strange parts of the brain. Coghill (1923) observed an obvious correlation between acceleration of growth in a region of the central nervous system and the ingrowth of axons into the region, and he suggested (1924*b*) the possibility that accelerated differentiation might account for the ingrowth of sensory fibres. However, Coghill (1926*c*, 1933*b*) could discover no actual correlation in either time or space between accelerated proliferation or differentiation and the point of origin of either dorsal or ventral spinal nerve roots in early amphibian cord development. Burr (1923*b*, 1924) grafted additional olfactory placodes adjacent to those of the host in *Amblystoma* and observed a number of cases in which aberrant olfactory fibres from the grafted organ entered either the midbrain or the pars dorsalis thalami. Burr explained this ectopic nerve entrance as due to a strong attractive stimulus of midbrain for olfactory nerve. Olfactory nerves will also enter the medulla (Piatt, 1947), hence the 'attraction' must be non-specific in nature. Burr (1932) observed later that local bursts of mitotic activity could be correlated in both time and space with the point of entrance of the various cranial nerves. He observed one such proliferation peak at the time and place in the brain wall that the aberrant olfactory nerves were seen to enter the midbrain. He concluded that these localized regions of mitoses were indicative of a high rate of metabolic activity at that particular time and place and occasioned bio-electrical phenomena which created the real attractive stimulus. This theory is intriguing but has as yet received no direct confirmation. Experiments by Detwiler (1929*c*) and Szepsenwol (1938) would appear to indicate that optic or olfactory nerves seldom enter ectopic parts of the brain.

Grafted ectopic cranial nerve roots (vestibular and lateral-line) in *Amblystoma* (Piatt, 1947) usually enter the brain at the same level as the predominant host nerve to which they become adherent. Although the cephalo-caudal level of entrance usually corresponds to host root entrance, the latter is often not the shortest route into the brain. Furthermore, the ectopic root fibres enter the precise dorso-ventral level to which they are normally related. It would appear that the particular antero-posterior site of entrance is determined by constant but non-specific factors while the point of dorso-ventral (medio-lateral) entrance is referable to more specific factors, i.e. vestibular and lateral-line fibres both enter at the Vth root level but the former enter the tractus acusticus and the latter the more dorsal lateral-line tracts. The data do not warrant any further conclusions.

IV. STRUCTURAL RECONSTITUTION AND REPAIR

Structural regulability, reconstitution and regeneration of the nervous system is much too vast a field for review here. A large-sized volume would be necessary for an adequate treatment of the subject. Lee (1929) reviewed the problem of nervous tissue regeneration almost 20 years ago; his incomplete bibliography included 272 titles, of which one, published in 1907, contained over 750 titles itself. The present account is limited almost entirely to embryonic reconstitution of the central nervous system, and chiefly to more recent experiments on amphibians.

Early experiments by Schaper (1898) showed that, when large portions of the embryonic brain were excised or cauterized, very little regeneration of the missing brain parts took place. Bell (1907) showed that, when younger embryos were employed and less radical injuries made, the embryonic amphibian brain demonstrates definite regenerative capacity. He noticed the interesting fact that, when a piece was removed, reconstitution took place chiefly from the lateral or medial wound surfaces and not from either cephalic or caudal ends. Lewis (1910) excised pieces from various regions of the neural plate in urodele and anuran embryos. The remaining part of the plate reconstituted a complete central nervous system in almost every case. Experiments by Adelmann (1929*b*) also demonstrate the equipotentiality of the neural plate. When excised pieces were transplanted heterotopically, even less than a lateral half had the power to form a bilaterally symmetrical structure. Bilateral symmetry does not necessarily imply complete histogenesis, however. If trunk neural folds of *Amblystoma* are removed (DuShane, 1938), Rohon-Beard cells are absent from the operated length of the spinal cord. When the medio-lateral axis of the future cord is reversed at stage 15, however, Hutchinson (1936) found some Rohon-Beard cells still occupying the dorsal (originally presumptive ventral region) part of the operated section of cord. When bilateral extirpation of the mesencephalic neural folds (probable source of mesencephalic V root cells) is performed (Piatt, 1945), no numerical deficit of mesencephalic V root cells is found. The results of these latter two experiments indicate that restitution of specific, specialized cellular elements is also possible in neural fold stages of development, certain neuroblasts apparently remaining isotropic during these earlier stages. Removal of one cerebral hemisphere in *Amblystoma* embryos resulted in complete restitution of the missing part if the nasal organ was intact (Burr, 1916*b*); regeneration of only the pallial portion if the nasal organ was excised also. Burr concluded that regeneration was dependent upon the stimulus derived from ingrowing nerve fibres, either peripheral or intracentral.

A recent series of experiments by Detwiler has demonstrated the great reconstitution and regulatory powers of the early central nervous system in amphibians. Detwiler performed unilateral (1940*b*) and bilateral (1943*a*) end for end reversals of the medulla at closed neural fold stages in *Amblystoma*. In both experiments the reversed portion reconstituted itself into a normal medulla showing progressive cephalo-caudal tapering and diminishing cephalo-caudal cell counts. The axon of

Mauthner's cell, when present, grew caudally in most cases, demonstrating a lack of polarity determination at the time of operation. End for end reversal of the entire mesencephalon (Detwiler, 1946*a*) gave a normal appearing mesencephalon with almost normal function; hence Detwiler concluded that the neuroblasts of the tecto-bulbar and tecto-spinal tracts are not yet polarized at this time (stage 22). No actual description is given of these tracts, however, and, in the light of Oppenheimer's (1945) conclusions on *Fundulus* with abnormal Mauthner's cell arrangements, it may be that normal function was obtained partly by functional plasticity rather than through a purely anatomical regulability. (See also Sperry above.) Unilateral reversal of the mesencephalon resulted, on the other hand, in many abnormalities, although the intact half often reconstituted itself into a fairly good whole in some cases. Despite the regulability of the medulla in reversed conditions, it shows a striking tendency to develop with characteristically medulla features when a lateral half is substituted for the corresponding half of the brachial region of the cord (Detwiler, 1943*b*).

Further experiments (Detwiler, 1944*b*, 1946*b*, 1947) have shown that unilateral extirpations of the medulla, midbrain or brachial region of the cord result in each instance in a complete restitution of the missing half. In almost all cases the central nervous system is essentially normal and bilaterally symmetrical through the operated regions. Analysis of the progress of these restitutions led Detwiler to conclude that almost all regeneration took place by an extensive proliferation and migration of extra-ependymal cells from the opposite, intact half, not from either cephalic or caudal stumps on the side of operation. Mauthner's cell was missing in all cases of medulla regeneration, indicating to Detwiler that the specificity of this neuron is determined prior to the developmental stages (21–23) used. In these cases the VIIth and VIIIth cranial roots were also absent, which may account, in part, for the non-differentiation of the cell (Piatt, 1947). Detwiler (1944*a*, 1945, 1946*c*) also performed bilateral and unilateral extirpation of the forebrain in neural fold stages of *Amblystoma*. Contrary to the positive cases of regeneration obtained after unilateral removal of medulla, mesencephalon or brachial cord he observed no regeneration of the cerebral hemispheres following either unilateral or bilateral excision. The nasal organ was removed along with the forebrain in these experiments and Detwiler agrees with Burr's earlier contention (see above) that cerebral hemisphere regeneration is dependent upon the presence of a nasal organ. In cases of unilateral excision the intact hemisphere underwent a compensatory increase in size. The medulla in the operated animals was normal and Detwiler concluded that the prosencephalon exerts no morphogenetic influence on the rest of the brain.

Studies on restitution of the spinal cord in early neurula and tail-bud stages of anurans and urodeles were made by Hooker (1915, 1923, 1925). Following complete transection of the cord in these early stages, both anatomical and physiological continuity may result. As pointed out by Hooker, regeneration of this type in the neurula stages is a process of simple healing and morphogenesis since few, if any, fibres are as yet present. He found, as did Wieman (see above), that descending neuraxes from the ventrolateral column invaded the wound region before ascending fibres from the dorsolateral column. Lorente de Nó (1921) transected spinal cords of older anurans (20–35 mm.) and obtained anatomical regeneration in most cases, but the neurons did not reproduce themselves at the cut ends of cord as did those in Hooker's experiments. Regeneration of the amphibian central nervous system, at least in the earlier stages, is well established.

Reconstitution of the early embryonic central nervous system in fish is apparently more limited than in urodeles and approaches to some extent a mosaic type of development. Lewis (1912) first showed that defects made on the nervous system of *Fundulus* embryos, particularly after the stage of embryonic shield formation, were usually permanent. Hoadley (1928) also showed that, when various presumptive regions of the nervous system were removed in *Fundulus* embryos of the embryonic shield stage, parts of the central nervous system were missing. Both of these earlier investigators concluded that the neural axis parts are determined and fixed at a relatively early stage in *Fundulus*. Nicholas & Oppenheimer (1942) have recently reinvestigated this problem in *Fundulus*. They find, in general, that in the embryonic shield stage defects in the nervous system are more readily obtained than in other systems, but that, when 15%, or less, of the shield material is removed, almost complete regulation occurs. The extent of damage is therefore important. In premotile stages, if a three-somites length of cord is removed (stages 17–20) or injury to the brain is done (stages 18–19) very little anatomical restitution takes place, and no physiological recovery in the instances of cord removal. On the other hand, transection of lower medulla or cord in later stages (motile) resulted in complete anatomical reconstitution. They explain this seemingly unorthodox observation—better restitution in later stages when it would be least expected—as due to the fact that interference in earlier stages with fundamental morphodynamic movements has more far-reaching effects; at later stages these morphodynamic forces have already accomplished the desired morphogenetic ends. It should be pointed out, though, that the extent and nature of the injuries performed in later stages were in no way as severe as those inflicted in the earlier ones. On this basis alone one would expect far less permanent damage to the central nervous system in the operations on later stages. In this connexion it is of interest to note that, whereas Hooker (1932) could obtain cord regeneration in 'adult' fish only when the transection was made during the first 4 days following hatching, Tuge & Hanzawa (1937) obtained morphological regeneration and functional recovery in full-grown adult fish. No explanation can be offered here for this discrepancy. Two different species of fish were used, but it appears doubtful whether this would account for the difference in results.

Defect experiments made on early chick embryos by Ferret & Weber (1904) resulted in various abnormalities of the central nervous system. Fugo (1940) extirpated most of the prosencephalon in chicks of 33–38 hr. incubation to eliminate development of the hypophysis. Little is said concerning amount of restitution of missing forebrain but it is assumed that very little, if any, took place. Rhines (1944) and Rhines & Windle (1944) also removed various regions of the early embryonic chick brain. They observed only slight reconstitution phenomena in most cases.

I am unaware of any experiments on reconstitution of the very early embryonic mammalian brain. Nicholas & Hooker (1928) and Hooker & Nicholas (1930) made a number of spinal cord transections of rat foetuses *in utero* and discovered no evidence of any regenerative phenomena. They concluded that vascular changes followed by absence of cellular replacement were the probable causes preventing regeneration. Gerard & Grinker (1931) performed complete and partial cord transections in new-born rats. They found some fibre regeneration across the gap; in some cases a gradual return of function ensued. They thought return of function to be a matter of physiological reorganization. Sugar & Gerard (1940) have more recently reported true anatomical and physiological regeneration following complete cord sections in 3–5 weeks old rats. In many of these successful cases, however, the gap was bridged by a piece of the sciatic nerve. Clark (1943) divided certain cranial and spinal nerves in the adult rabbit and inserted either cut proximal or distal stumps into the brain; the brain nerve fibres gave no evidence of regeneration. Only tentative explanations for failure of brain regeneration were offered by him. Recently May (1946) transplanted new-born cerebral tissue and pieces of sciatic nerve to the anterior chamber of the eye in adult mice. In many cases the grafted cerebral tissue grew toward and regenerated in the grafted nerve segment. May concluded that cerebral cells possess the same inherent capacity for regeneration as do peripheral fibres. He also thought the degenerating grafted nerve pieces attracted the fibres from the young cerebral neurons. This latter conclusion is interesting in view of unpublished experiments of the writer on *Amblystoma*. Adult nerve segments transplanted into or near embryonic central nervous tissue eventually disappeared completely and gave not the slightest indication of having influenced or directed fibre growth of intramedullary neurons. In a recent review Young (1942) has pointed out that no inherent reasons are known to exist which would preclude the regeneration of mammalian central nervous tissue but that lack of central restitution is probably due to special factors. Recent studies (Weiss, 1944*a*, *b*; Weiss, Wang, Taylor & Edds, 1945) offer substantial evidence that the nerve fibre itself (peripheral in these experiments) is undergoing continuous growth during its entire adult existence.

V. GENERAL CONCLUSIONS

The problem of neural plate determination is inextricably linked with the more general field of embryonic induction. It affords an excellent example of using the neural axis as a tool, an indicator rather than a subject of study within itself. It appears clear that the neural plate is dependent upon the presence of chorda-mesoderm for its normal development. It is not clear, however, at what point this influence may cease to be necessary or even effective. Neurulations reported without benefit of mesodermal substratum are probably the results of pathological conditions and are not critical in demonstrating independent differentiation of presumptive neural ectoderm. There is an increasing amount of evidence, on the other hand, that neural tissue once inducted is capable of more different modes of self-differentiation than previously supposed. There is strong evidence that morphological gradients of the neural axis are inherent within the neural ectoderm itself, and, although there exists as yet no critical proof that these gradients are not imposed from without during the very early stages of gastrulation, it is becoming increasingly clear that any such supposed effect is rapidly superseded by purely ectodermal forces. The formation of a rounded neural tube from a flat plate of cells is largely independent of extrinsic forces. Purely physical forces are thought to be predominant, but the precise nature and implementation of these forces are not understood. Differential water absorption and cellular proliferations are probably not instrumental in the process of tube formation. Our knowledge of the causal relationship of earliest nervous system formation have been derived chiefly from a study of amphibian material. General similarity in all vertebrate groups is undoubtedly the case; it still remains to point out the differences which may obtain.

There exists no valid evidence that true metamerism is inherent in the early neural axis. Neuromeres which delighted the earlier neuroembryologists can be correlated neither with the future segmentation of the central nervous system nor with mesodermal segmentation, and hence lose most of their significance. Mesodermal segmentation is primary and experimental data have shown that spinal nerve and ganglion segmentation is a secondary acquisition imposed from without by virtue of the segmentation of the axial mesoderm. This phase of central nervous system organization appears to be less independent of external forces than late neural plate and tube formation.

The development of specific elements and their structural relationships within the central nervous system cannot be adequately explained by the theories of either neurobiotaxis or stimulogenous fibrillation. Neither of these theories has as yet received direct experimental proof; on the contrary considerable evidence has accumulated against them. It is the opinion of the reviewer that unless they can carry their own weight, they should be allowed to die a natural death.

Almost nothing is really known concerning those factors which initiate and control cellular proliferation and differentiation within the central nervous system. The distinction between cell increase and cell differentiation is an important one, but also one which has often been ignored. The two processes are not only referable to different causes but are mutually exclusive. Both processes work toward the same end, however, and in this general sense they are alike. The role of longitudinal fibre tracts in stimulating either proliferation or differentiation of neuroblasts has probably been over-emphasized in the past. There is evidence that cell number within the various parts of the neural axis is controlled to some extent by a position effect with reference to the anterior-posterior gradient, but the more anterior regions seem to possess a greater inherent capacity for self-development than do the more posterior. There is some evidence that indifferent cells are caused to differentiate by invasion of their territory by neighbouring cell processes. This belief lacks critical experimental proof. Definitive cell numbers in both cranio-spinal ganglia and sensory and motor areas within the neural axis are regulated to a large extent by the size of their respective peripheral fields. This is true for all major groups of vertebrates studied, including urodeles. There is experimental evidence that this control is implemented during the later differentiation phase of development rather than in the earlier proliferation phase, but more evidence is needed. The actual mechanism whereby non-nervous tissue effects this control of neuroblasts is not known. Several theories are on record but only two of the more recent explanations carry much weight. One of these would place control at the initial phase of differentiation; the other indicates an atrophy of already differentiated cells. Much of the more recent evidence assigns to peripheral factors the predominant role in regulating cell number within the central nervous system, rather than intracentral factors. With respect to purely differentiation phenomena this may prove to be the case; with regard to proliferation of indifferent cells intracentral factors are probably more important. There is no valid reason to believe that central nervous tissue itself does not possess great inherent capacity both for proliferation and differentiation.

The study of directional nerve growth has been done almost entirely on the peripheral nervous system. The developmental reasons for specific nerve patterns and polarity of growth within the central nervous system are unknown. Some recent experiments have assigned to longitudinal fibre tracts a directing influence upon decussation and polarity of fibre growth. Other experiments have failed to substantiate such claims and deny that pre-existing fibre tracts influence in any way the directional growth of intramedullary axons. Regardless of which view may eventually prove to be more correct, polarity of growth in these very earliest tracts still remains to be explained. It appears most improbable that growing nerve centres and their contingent of nerve fibres exert no guiding influence upon neighbouring centres, but up to the present time no positive, well-tested data in support of such a contention are available. Much of the older work which purported to have proved dynamic, directional growth stimuli operating within the central nervous system is now seriously open to question. The behaviour of growing intramedullary nerve fibres and the routes they have been observed to follow in various experiments cannot be explained, however, on the basis of purely mechanical factors or substrate configurations. Further research along these lines is badly needed.

There is some indication that regions of high metabolic activity within the central nervous system can be correlated with the point of entrance of the cranio-spinal nerves. Much more evidence is needed to prove such a thesis. The entrance of ectopic cranial nerves into the brain takes place usually at loci where normally situated nerves enter. The precise medio-lateral point of entrance depends upon the kind of fibres entering. There is considerable evidence that centripetally directed cranial nerves are directed to particular medio-lateral points of the brain wall by forces of a specific nature which are probably correlated with functional homogeneity of nerve and brain centre.

Structural regulability of the early central nervous system reaches a high degree in amphibians. Other vertebrate groups, such as fishes, manifest less reconstitution phenomena. Specific types of highly differentiated cells are probably determined at relatively early stages of development but may still be replaced by indifferent cells at comparatively late stages. The histogenesis of the various cell types is probably, therefore, controlled by external conditions as well as inherent differentiation proclivities. Good structural and functional regeneration of intramedullary fibres is possible, just as in peripheral nerves. In older developmental stages and in the higher classes of vertebrates special inhibiting factors appear to be present which prevent extensive regeneration within the central nervous system. Almost nothing is known concerning the amount of structural repair possible in mammalian central nervous tissue during the earliest stages.

The chief desiderata in the study of causal neurogenesis of the central nervous system are: (1) a working hypothesis to be used in further analyses of differentiation phenomena; (2) more experimental studies concerned specifically with causal relationships directing the growth of intramedullary fibres and tracts. Much more work is required along these lines before it will be possible to establish sound principles of differentiation and growth which will serve to make more comprehensible the anatomical and functional significance of the different elements composing the central nervous system. The over-all difficulty has been the lack of conscious and co-ordinated approach toward these problems and the incidental character of the data.

VI. SUMMARY

The causal relationships responsible for the early organization and subsequent structural configuration of the vertebrate central nervous system are poorly understood. This is due in part to the complex nature of the subject but perhaps even more to the fact that causal neurogenesis has not yet attained full status as an independent biological discipline. Those general principles which are known derive largely from the separate fields of experimental embryology and descriptive neurology and are based, for the most part, on a random collection of non-integrated facts. The source and nature of the data are probably adequate; what is needed is a conscious, co-ordinated effort to envisage this field of study in its totality and, for immediate purposes, as an end in itself.

1. The early formation of the medullary plate is dependent upon the inductive effect of the mesodermal substratum. Physical contact is probably necessary. Organization of neural ectoderm into medullary plate and tube in the absence of underlying tissue has been reported, but these results are probably due to the disintegrating action of the culture medium upon the inner, uncoated plate cells. The autolysis produced liberates neurogenetic substances responsible for the inductions obtained and the process is considered a pathological one. The morphogenetic forces responsible for the folding of a flat medullary plate into a hollow tube are probably intrinsic to the neural ectoderm itself. An increase in the contractile tension of the superficial gel layer of the external ends of the plate cells may be an important factor. Neither differential water absorption nor localized cellular proliferation seems to play a part in the process. The establishment of polarity and morphological gradients within the neural axis are relatively independent of substrate tissue. Throughout the early stages of neurogenesis the neural ectoderm plays a more active role than has previously been supposed.

2. There exists no valid evidence that neuromeres represent an inherent metamerism of the early neural axis. The segmental arrangement of the spinal ganglia, and probably

dorsal and ventral roots as well, is imposed from without by virtue of the proximity of the segmenting axial mesoderm. Mesodermal segmentation is primary; ectodermal segmentation is secondary. The organizing effect of the mesoderm upon the segmentation of the neural tube is more pronounced than certain aspects of the mesodermal-ectodermal relationship obtaining in earlier stages.

3. The theories of Kappers (neurobiotaxis) and of Bok (stimulogenous fibrillation) are inadequate for solving the complexities of neural differentiation. Neither theory is buttressed by critical experimental evidence and both are highly speculative in nature. Neurobiotaxis and stimulogenous fibrillation still remain the *dues ex machina* of neurology.

4. Nothing decisive is known concerning the important phenomena of cellular proliferation and differentiation within the central nervous system. Failure to delineate clearly between these two processes has caused some confusion in the past. Cell number is controlled to some extent by position of a part within the anterior-posterior gradient; more anterior regions of the neural tube are less dependent upon such a position effect. The definitive number of differentiated cells constituting a specific region is regulated by the size of the peripheral field. This is true of intramedullary as well as extramedullary cells and is common to all major vertebrate groups studied. The mechanism whereby non-nervous tissue effects this control of neuroblasts is not known. Experimental studies differ in their interpretation as to whether regulation occurs during the initial stage of differentiation or is implemented through the subsequent atrophy of already differentiated cells. Invasion of an undifferentiated field by nerve fibres from a neighbouring region has been thought to initiate the differentiation of the indifferent neuroblasts. Critical evidence for such a thesis is as yet lacking. There is good experimental reason to believe that the non-nervous periphery affects differentiation alone, while proliferation of indifferent cells is influenced to a great extent by intracentral factors.

5. The arrangements of nerve fibres into specific tracts and the polarity of their growth is generally supposed to be influenced by pre-existing fibre pathways, particularly the fasciculus longitudinalis medialis. Regenerating intramedullary fibres have been observed to traverse preferential routes through the embryonic cord and to exhibit marked polarity in their growth. Substrate configuration and mechanical forces are probably of great importance in directing fibre growth. The reason for the formation of the standard commissures and fibre decussations is not known. The operation of purely mechanical forces within the central nervous system appears inadequate to explain all the intricacies of fibre pattern observed.

6. The precise point of entrance of sensory components of cranial and spinal nerves into the central nervous system has been thought to be determined by localized attraction centres of high metabolic activity. Indirect evidence exists for such a supposition but critical proof is lacking. Ectopic sensory nerve roots usually enter the brain wall at definite loci and their dorso-ventral (medio-lateral) orientation is probably determined by the position of correlation tracts of corresponding function. The place of exit of motor roots can be experimentally altered, but nothing is known concerning the causal relationships involved in their normal egress.

7. Structural regulability of the embryonic central nervous system reaches a high level in amphibians, less so in fishes and mammals. Specialized cell types are determined early in ontogeny but may be replaced from indifferent cells at relatively late stages. The cytogenesis of various cell types is probably influenced by factors extrinsic to the cells themselves. Anatomical and functional repair of intramedullary fibres is possible. Special inhibiting factors are probably responsible for failure of proper regeneration rather than any inherent difference between central and peripheral nervous tissue.

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ADDENDUM

Since the completion of this article an interesting communication (in litt.) has been received from Dr G. Fankhauser, Princeton University. This worker has found bilateral duplication of Mauthner's cell in a haploid larva of *Triturus*. Fankhauser in several previous publications has shown that cell number varies inversely with the degree of ploidy and he investigated the haploid animals with this in mind. No strict correlation between haploidy and double Mauthner's cells can be made until more cases are examined, but Fankhauser's observation may afford another clue to the problems of cellular proliferation and differentiation.

Attention may also be drawn to two important papers of recent date. R. G. Harrison (1947, 'Wound healing and reconstitution of the central nervous system of the amphibian embryo after removal of parts of the neural plate', *J. Exp. Zool.* **106**, 27) has shown that extirpated lateral halves or parts of the neural plate are restored chiefly from the contralateral intact side through two processes: (1) transference of 12 to 22% of the original intact half to the defective side by secondary formation of the central longitudinal sulcus and subsequent rotation of the neural tube; (2) extensive cellular hyperplasia on the regenerating side which is relatively greater than the intact side in early stages and absolutely greater in later stages. A. M. Dalcq (1946, 'Recent experimental contributions to brain morphogenesis in amphibians', *Growth*, **10**, Suppl. 69) has written a provocative summary of experiments performed by himself and others on the induction and regional differentiation of the amphibian central nervous system. He believes that the unity of the cerebro-spinal axis is only apparent; the neural plate actually results from the combined inductive effect of several mesodermal substrata. Also, the prechordal plate is not subdivided into special evocators for definitive forebrain parts but these latter arise through the interaction of other formative forces at higher levels of induction. Both of these papers require more discussion than can be given here.

TAXONOMIC PROBLEMS IN THE EUGLENINEAE

By E. G. PRINGSHEIM
Botany School, University of Cambridge

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I. INTRODUCTION

Since Klebs (1883) laid the foundations of our knowledge of the Euglenineae many details have been accumulated and the number of species has grown very considerably, so that it is a matter of urgency to bring some order into this mass of facts.

More or less closely following Klebs most authors based their treatment of the group on the separation of the green forms as the Euglenaceae from the unpigmented ones, dividing the latter into the saprotrophic Astasiaceae and the zootrophic Peranemaceae. 'Although this classification' is 'no doubt artificial, it affords a convenient synopsis' (Fritsch, 1935, p. 741; cf. also Jahn, 1946, p. 264). Klebs, seeing clearly that some of the Astasiaceae are very near to some species of the Euglenaceae, and that the difference in pigmentation does not suffice to separate large taxonomic groups, tried to use the circumstances during cell-division as a further means of separating them, *Euglena* being supposed to divide in a non-motile, *Astasia* in a motile state.

Further progress depended on a more detailed study of cell-morphology to which French and American authors have contributed a great deal.

Chadefaud (1937) published studies of the cell contents of the Euglenineae, Hollande (1942) followed with an elaborate investigation of certain flagellates. Both throw light on euglenoid structures essential from a taxonomical point of view. Hollande came to the conclusion that the species of *Astasia* are to be considered as irreversibly apoplastidic *Euglenae* (p. 141). The paragraph on the phylogeny and classification of the Euglenineae, though not sufficiently taking notice of established facts, gives a valuable attempt at a more natural classification than had been published up to then. Hollande discerns five families: the Eutreptiidae, Distigmidae, Euglenidae, Menoidiidae and Peranemidae. The Eutreptiidae and Distigmidae should be united in one group (see below), in the same way as the Euglenaceae and the Astasiaceae are, according to Hollande, to form the Euglenidae. He characterizes the Menoidiidae by a non-bifurcated flagellum, a doubtfully established feature.

The review by Jahn (1946) is entirely devoted to the Euglenineae and gives a very useful survey of the relevant characters with many details of taxonomical importance. The evolution of *Astasia* from *Euglena* is briefly discussed, the relation of the Astasiidae to the Peranemidae in more detail. By using the creeping movement as a criterion Jahn believes *Distigma* could perhaps be placed in the Peranemidae, forgetting that a number of green forms exhibit the same kind of locomotion, but he is right in saying 'One way out of the dilemma would be to have only one colourless family instead of two, but this would result in an unusual diversity within the family' (p. 265). It would even be worse than he realizes because the distinction between the green and the colourless forms is still less secure. Jahn divides the Euglenineae into the families Euglenidae, Colaciidae, Astasiidae and Peranemidae. The Colaciidae differ from the Euglenidae by their palmelloid development and by the supposed single flagellum without bifurcation (Johnson, 1934). The latter statement should not be accepted without further proof.

The papers of Hollande (1942) and Jahn (1946), though both together affording a valuable review and adding much to our knowledge of the Euglenineae, are largely written from a zoological point of view. The present article is intended to supplement them, mainly along lines more relevant to a botanist.

The Euglenineae are a very well-defined class, unrelated to other flagellate classes. None of them exhibits a truly algal organization as characterized by rigid cell walls, composed of substances different from the protoplasm, and by a non-motile, mostly sessile habit.

Characteristic features are: the peculiar, often twisted and therefore asymmetrical shape of the cell; the 'ciliated' flagella, bearing numerous fine lashes; the grass-green colour of the chromatophores; the large vacuolar reservoir and canal (often misleadingly called gullet and pharynx), into which the contractile vacuoles shed their contents; the absence of sexual reproduction; and the starch-like reserve substance, paramylon.

Diversity within the class may affect the nature of the periplast, whether rigid, or soft, and allowing considerable euglenoid or metabolic changes of shape; the production of envelopes; the shapes and numbers of chromatophores or their absence; the gliding movements; the mode of nutrition.

The subdivision of the class into taxonomic groups is a matter of controversy. This is partly due to insufficient knowledge—especially of cytological details but also of nutritional features—and partly to the existence of transitional forms. Genera are few, but species are many. The definition of the latter is not always adequate, owing to pronounced variation in appearance under different environmental conditions. The lack of simple cultural methods and of good figures has hampered progress in the study of this group of relatively large and robust flagellates, in spite of the attention they have received.

Many species of Euglenineae are abundant, especially in waters polluted with animal excrements; others are planktonic or occur in isolated specimens among rich flagellate and algal populations. Their ecological distribution is such that they are potentially valuable indicators of water conditions, given trustworthy identification. However, in the long lists of names found in floristic and ecological papers many are of doubtful validity because existing diagnoses and figures are inadequate to ensure reliable determination.

In the future it is urged that larger and more detailed figures than are usual now be given, so that the cell contents are distinctly shown. It should be stated in what respects a given material conforms to, or differs from, earlier descriptions, and it should be made clear why it is identified with the species named. It would also be valuable to learn under what ecological conditions, at what time of the year, and in what community of other organisms newly described or reinvestigated species have been observed.

II. TAXONOMIC FEATURES

(1) *Cell shape and size*

The genus *Euglena* has always more or less consciously been taken as the prototype of the Euglenineae. Nevertheless, the basic form of the euglenoid cell has not been grasped: it is neither symmetrical nor radial, but helical, and there are therefore no planes dividing the cell into equal halves. In *Lepocinclis* and *Trachelomonas* there are species with almost radial symmetry, but this is apparently secondary, the basic asymmetry being still revealed by the position of the eye-spot; it is not situated in the median plane defined in *Lepocinclis* by the paramylon rings and in *Trachelomonas volvocina* by the pyrenoids.

Despite its metaboly the euglenoid cell is generally spindle-shaped. On closer inspection most of the cells show a periodic tapering towards the posterior end (Fig. 1). The common tail-like process, mentioned in many diagnoses, is only the last of these constrictions. Almost every *Euglena*, and some *Phacus* and *Astasia* species, have a wavy outline. This seems to be caused by a screw-like torsion of the cell (Fig. 2)* and can be imitated by twisting an elastic model. The periodic tapering is seen not only in species with a rigid periplast but also in highly metabolic *Euglena* and *Astasia* species and in the naked soft cells of *Trachelomonas* (Fig. 3). Many species, differing more from the hypothetical ancestors, do not show clearly the basic euglenoid shape. The dimensions of the cells are commonly used for diagnostic purposes and are sometimes almost the only features given in floristic surveys. This practice is often misleading, either because the dimensions cannot exactly be given, or because they are not characteristic of a species. The former difficulty arises in metabolic species. Without fixation the cells cannot be measured, and fixation may considerably alter the width and length of the cells. Moreover, there is no means of knowing which is the typical form to be measured. The volume of the cells seems to be constant, but their irregular shape does not permit exact calculation.

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* More examples are found in Pochmann (1942).

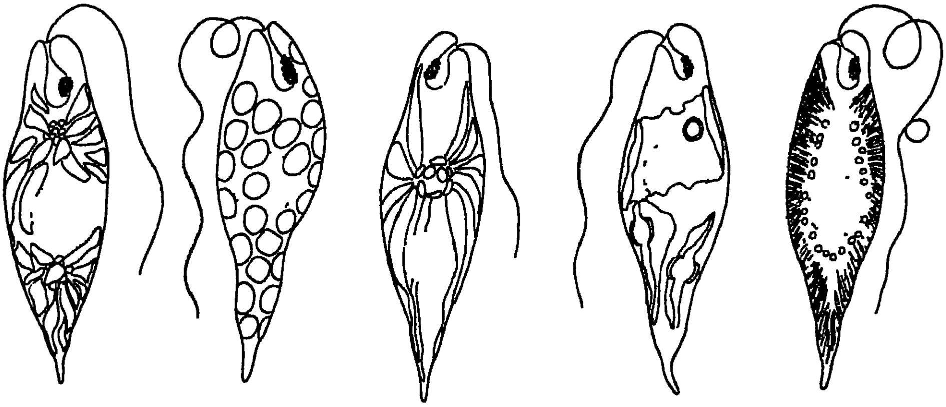

Fig. 1. Several *Euglena* species showing: (1) similar shapes; (2) periodic tapering of the posterior region; (3) different types of chromatophore structure. *a*, *Euglena geniculata*; *b*, *E. variabilis*; *c*, *E. viridis*; *d*, *E. anabaena*; *e*, *E. rubida*.

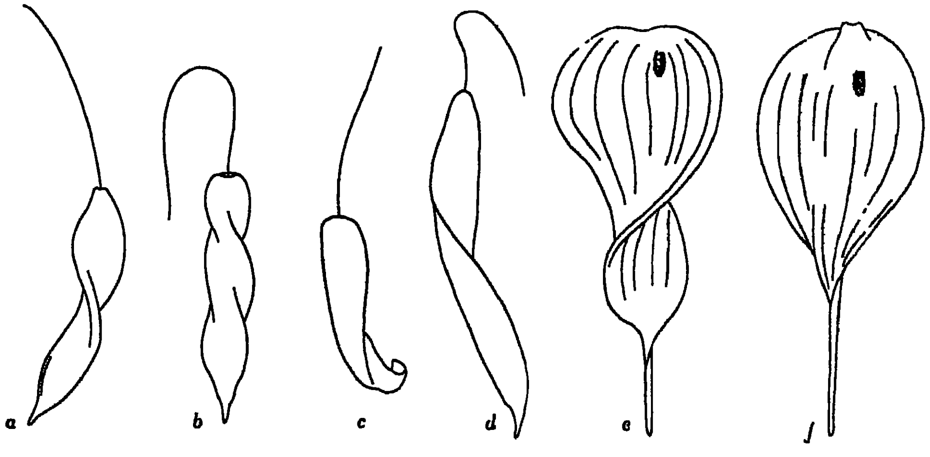

Fig. 2. Tendency towards spiral body form in some Euglenineae: *a*, *Astasia torta*; *b*, *A. granulata*; *c*, *Rhabdomonas spiralis*; *d*, *Euglena spirogyra*; *e*, *f*, *Phacus tortus*—two views.

The latter, more serious, difficulty is due to the existence of varieties differing in size, especially in *Euglena* and *Distigma* spp., e.g. *Euglena agilis* Carter (syn. *pisciformis* Klebs), *E. anabaena* Mainx, *E. deses* Ehrbg., *Distigma curvata* Pringsh. It is not known whether these dimensional differences are connected with nuclear differences, for instance in the number of chromosomes. They are often linked with differences in the shape of the cell and the number and shape of the chromatophores.

Further, neither the size nor the shape of the body are so different in many species of *Euglena* that a natural classification or a diagnostic key can be based on them, nor are they sufficiently constant for these purposes even in a genetically homogeneous material. While the cells of clone cultures are almost identical under reasonably similar conditions, slight differences in these conditions alter their appearance considerably. Moreover, there are varieties, constantly differing from the 'type' and from one another, which, though they should be kept together in collective species (*E. agilis*, *E. anabaena*, *E. deses*, *E. viridis*, etc.), would be separated, if judged only by their shapes and sizes. In the *E. acus* group many forms differing in dimensions have been given names and newly observed ones can usually be interpolated between those already described. The variation affects not only the size of the body but also the ratio of length to width. In *E. agilis* there is a variety which, in external form and distribution of chloroplasts and nucleus, simulates *E. viridis* and under low power magnification may deceive the observer.

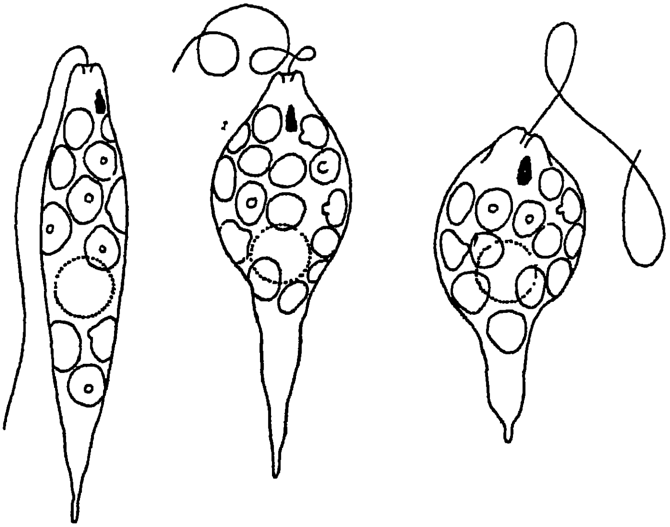

Fig. 3. Naked cells of *Trachelomonas conspersa* showing strong metaboly, periodic tapering, and relative length of flagellum.

Several *Euglena* species have a very similar shape, for instance *E. geniculata*, *E. proxima*, *E. viridis*, *E. anabaena*, *E. rubida*, *E. magnifica* n.sp., *E. sociabilis*, *E. splendens*, etc. (Fig. 1) and cannot therefore be identified on this ground alone. The only way out of the difficulty is to use as many features for identification and differentiation as possible. The nature of the plastidome, i.e. the number and shape of the chromatophores and the features supplied by the pyrenoids, are of special value. Description of *Euglena* species is impossible without reference to these characteristics. Johnson (1944), for instance, described as *E. viridis* a species which, as shown in his fig. 37E (p. 131), possesses double-sheathed pyrenoids.

(2) *Cell organs*

(*a*) *Nucleus*. All euglenoid nuclei seem to have the same main characteristics (cf. Hollande (1942) and Jahn (1946)). There is a caryosome, like the nucleolus of many other organisms, but only seen after fixation and persisting during nuclear division. The nucleus of the living cell appears as a homogeneous limpid body and is generally spherical, but elongated in the more slender species *E. deses*, *E. mutabilis*, *E. acus* and especially in *Astasia linealis* (Pringsheim, 1942). The position of the nucleus in the cell is usually central, never anterior, but in some species, for example *Euglena viridis*, *E. agilis* and frequently in *E. gracilis*, it is posterior. It lies near the posterior end in *Trachelomonas*, *Lepocinclis* and *Cryptoglena*.

(*b*) *Chromatophores and pyrenoids*. Though these structures exhibit such a marked diversity within the class there are certain general rules concerning their distribution. While the species of *Euglena* differ widely from each other in the composition of the plastidome, presenting every variety occurring in the entire class, there is no such diversity within the other genera. In *Phacus* and *Lepocinclis* the chromatophores are always true chloroplasts, very similar in shape and lacking pyrenoids, to those of higher plants. In *Trachelomonas* and *Colacium* they are larger, their number apparently depending on the space inside the cell, and each possesses a single pyrenoid. *Eutreptia viridis* has small discoid chromatophores without pyrenoids. Other species of this genus do not seem to have been sufficiently investigated in this respect.

In *Euglena* the small lenticular chromatophores or chloroplasts, found in the relatively rigid species *E. spirogyra*, *E. tripteris*, *E. acus*, etc., and in the metabolic species *E. Ehrenbergii*, *E. intermedia*, *E. variabilis*, *E. proxima*, are devoid of pyrenoids. The somewhat larger discoid chromatophores of the species *E. deses*, *E. Mesnilii* and *E. mutabilis*, which clearly form another natural group, have naked pyrenoids, one in each chromatophore. A large group, characterized by still bigger, often polyhedral or lobed chromatophores, has the characteristic conspicuous double-sheathed pyrenoids, as found in *E. agilis*, *E. gracilis*, *E. anabaena*, *E. granulata*, *E. obtusa* Schmitz (syn. *E. limosa* Gard), *E. sociabilis*, *E. polymorpha*, etc. Some, for example *E. sanguinea* and *E. magnifica* n.sp., have a rather complex chromatophore structure, such that the relation between chromatophores and pyrenoids is difficult to determine. The central part of the cell in these species is devoid of chromatophores. The green structures aggregate in a layer around this central space from which lobes radiate and tend to bend tangentially at the surface.

Complicated plastidome structures but without pyrenoids are found in two further groups of the genus *Euglena*. One possesses ribbon-shaped chromatophores, radiating from the centre around which paramylon grains are grouped so that a 'paramylon-herd', somewhat reminiscent of the paramylon sheaths of pyrenoids, is formed (Fig. 1*c*). To this group belong *E. viridis*, *E. geniculata* and *E. tristellata* Chu.

The other group has still more complicated chromatophores; the surface appearance differs from that at lower depth of focus and the various parts of the structure are difficult to make out owing to their overlapping and optical intermingling, e.g. in *E. rubida*, *E. splendens*, *E. truncata* n.sp., etc. The central part of the body is free of chromatophores, while the outer zone contains more or less radiating fringes. Just beneath the surface lie ribbon-shaped or elongate chromatophore portions arranged in spirals parallel to the striation of the periplast. This type is sometimes so similar to that of certain species with pyrenoids that it is not readily distinguished from them. The pyrenoids, however, are a constant distinctive feature and therefore of taxonomic value.

From their rigid periplasts and the character of their chromatophores, it would seem then that *Phacus* and *Lepocinclis* are nearest to the *Euglena tripteris* group; *Eutreptia*, from its metaboly and characteristic chromatophores to the *Euglena proxima* group; and *Trachelomonas*, from its discoidal chromatophores with double-sheathed pyrenoids and the type of metaboly, closest to the *Euglena anabaena* group, although the naked *Trachelomonas* cell (Fig. 3) is absolutely distinct from any form of the latter. The extraordinary length of the flagellum of *Trachelomonas* might be regarded as a resemblance to *Euglena polymorpha* which is likewise conspicuous by its flagellar length. In the naked expanded cells, however, this is much less pronounced, and in this condition the body of most of the species more closely resembles *E. anabaena* than *E. polymorpha*.

Confronted with such variety of plastidome structure in the Euglenineae one is tempted to ask which form is nearest to the common ancestor. Mainx (1927, p. 351) believed *E. gracilis* to be the most primitive species of this genus. The large rounded chromatophores with double-sheathed pyrenoids and the physiological behaviour of this species are in favour of Mainx's hypothesis, but the number of chromatophores is perhaps against it. Judged by other primitive algal types, the primitive *Euglena* should have a single, more or less cup-shaped, chromatophore.

In some algal groups the primitive character of archiplastidial forms with a single chromatophore is well established (Pascher, 1927, 1932, 1939; Vischer, 1945), but not in all of them, and in the Euglenineae the position is by no means clear. Chadefaud (1937) has described his *E. archaeplastidiata* as the most primitive form without pursuing the matter further or discussing the interrelationships of other species of *Euglena*. The organization of *E. archaeplastidiata* is in every respect, except its single chromatophore, like that of *E. agilis* Carter (syn. *E. pisciformis* Klebs), including the two pyrenoids characteristic of that species. As the number of pyrenoids in the Euglenineae is generally one only in each chromatophore, Chadefaud's form cannot be regarded, without more convincing evidence, as really primitive. It may, however, represent the closest living representation of the type from which *E. gracilis* and others can theoretically be derived. Further investigation is necessary. In contrast to Chadefaud, Mainx considers small numbers of chromatophores (*E. agilis*, *E. mutabilis*) as the derived condition compared with the case of *E. gracilis*, and there is nothing against this point of view. In the arrangement of the other species I agree with Mainx.

(*c*) *Flagellar apparatus and eye-spot*. The majority of the Euglenineae are known to possess one, two or, in some parasitic forms, more than two flagella. Since Wager (1900) showed that the flagellum of *E. viridis* is bifurcated within the reservoir and attached to its bottom by two 'roots' each terminating in a blepharoplast, more and more species have been found to possess the same kind of flagellar organization.

As is generally known, Hall & Jahn (1929) and Jahn & Kibben (1937) have stated that members of the family Astasiaceae, i.e. species of *Astasia* and *Menoidium*, differ from *Euglena* in possessing a simple unbifurcated flagellum. Lackey (1934) was the first to assert that an *Astasia* species had a bifurcated flagellum, and Hollande (1942) found the same in two more species. It is therefore doubtful if any member of the Astasiaceae really differs from the Euglenaceae in this respect. Those forms with an eye-spot, comprising the *A. quartana* group, have undoubtedly two flagellar insertions like the green members of the class, as already stated by Jahn and Kibben and confirmed by Hollande (1942).

The existence and the origin, ontogenetically and phylogenetically, of a bifurcated flagellum (if this description is to be taken literally) is rather difficult to imagine, especially as there are related forms, such as *Eutreptia* (comprising *Eutreptiella* Schiller and *Gymnastica* Pascher) and *Distigma*, which have two flagella, each attached to a single blepharoplast. In the light of these facts it seems on the whole more reasonable to consider the flagellar apparatus of the 'monoflagellate' forms as consisting likewise of two flagella. One of the 'roots' is thicker than the other and bears a swelling (the paraflagellar organ of Hollande (1942, p. 90)) and likely to be the light sensitive organ* (Mast, 1938, p. 195), while *Eutreptia* has such a structure on each of the two flagella.

In its non-flagellate state *Euglena mutabilis* has been shown by Hollande (1942, p. 88) to have two independent flagellar stumps, the relatively thicker and longer one bearing the enlargement and just reaching the outer opening of the funnel, the thinner being still shorter. As Mainx (1927) has observed that his *E. Klebsii* (identical with *E. mutabilis* Schmitz) swims freely after transference to a fresh medium, the thicker of Hollande's two stumps is presumably the one which, under certain circumstances, grows into an active flagellum. It is probable that other species without flagellar motility during a part (*E. deses*, *E. Mesnilii*) or the whole (*E. obtusa* Schmitz, syn. *E. limosa* Gard, *E. vermiformis*, *E. Ehrenbergii*) of their lives possess similar flagellar stumps.

The relation of the lenticular flagellar thickening to the point of bifurcation is given by various authors as either anterior or posterior to, or just at, this point. This uncertainty makes it still more difficult to believe in a real branching which one might expect to have a definite morphological significance, each part having its strict location. It is, however, just what one would expect to happen were there two independent flagella, one of them bearing the enlargement, meeting or cohering at an undefined point.

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* Two facts are in favour of this functional explanation. The swelling is not found in species without an eye-spot, and its shading by the latter causes the flagellum to beat sideways so that the course is altered.

The difficulty of reconciling the concept of flagellar bifurcation with actual observations is clearly demonstrated in Lackey's (1934) study of cell division in *Astasia* and *Distigma*. In both forms the division of the blepharoplasts is the first step to occur. As can be inferred from Lackey's figures one daughter blepharoplast forms a new short, the other a new long, flagellum, the production of short or long flagella being an inherent property of the relevant blepharoplast and its descendants. The two daughter blepharoplasts of the same kind separate, while the unlike pairs associate. This is quite obvious in *Distigma*, where no bifurcation or cohesion between the two flagella exists; but the phenomena in *Astasia* are so similar, and the figures of the two species supplement one another so well, that one cannot suppose there is any essential difference between the two cases.

Lackey was quite consciously aware that the concept of bifurcation is unintelligible. He says, for instance (p. 152), 'Evidently the roots of the new flagellum growing out from the daughter blepharoplasts unite after they have grown out a short distance', thus making it clear that he does not believe in bifurcation but rather in a secondary attachment of the two flagellar 'roots'. But he was too much under the impression of earlier papers to draw all the consequences. Although he showed that the flagella do not multiply by splitting along their length, but by the new one growing out of the blepharoplast, he assumes in his phylogenetic speculations that such a division of the flagellum itself is possible (p. 160). As an alternative he suggests that 'failure of the two roots to unite' might have given rise to the biflagellate colourless forms, thus indicating once more his tendency to regard the 'roots' as two independent flagellar units.

If the two 'roots' are actually two independent flagella, though perhaps sometimes coherent, one in an active and one in a rudimentary state, then the whole situation is more easily comprehended and, what is more, the homogeneity of the whole class is established. *Eutreptia* would be taken as the basic type in respect to the flagellar apparatus. It has not, as Hollande (1942, p. 183) believes, two equal flagella. In *Eutreptia viridis*, it is true, the two are of almost the same length, though one of them is stretched forward, the other sideways, and they act in a different way. In other species the two flagella are of different length, and they have therefore been given the generic names *Eutreptiella* and *Gymnastica*. By multiplication of the flagella such a form as *Euglenamorpha* can be derived from *Eutreptia*, and by the reduction of one flagellum to a subordinate or functionless role, the flagellar organization of *Euglena*.

Essentially the same organization is present in the colourless forms possessing an eye-spot, while its loss is combined with the loss of the flagellar swelling. The disappearance of the eye-spot and swelling is often, though not generally, connected with the loss of the chromatophores. In this way *Distigma* may have originated from *Eutreptia*-like ancestors in the same way as *Astasia* from *Euglena* (Fig. 4).

(*d*) *Periplast, metaboly and envelopes.* The periplast, or covering protoplasmic layer of the cell body, is soft in *Eutreptia viridis*, in most species of *Distigma*, and to a slightly lesser degree, in those of *Astasia*. In *Euglena* it varies much in rigidity, being in *E. mutabilis* almost as plastic as is general in *Astasia*, somewhat less flexible in *Euglena agilis*, *E. anabaena*, *E. deses*, *E. gracilis*, etc., and more rigid still in *E. acus*, *E. tripteris*, *E. pyrum*, etc. In the green genera *Lepocinclis* and *Phacus* and the colourless *Menoidium* and *Rhabdomonas* the periplast is rigid. That is, it does not yield to internal forces so that its shape is constantly changing. In *Trachelomonas*, some species of which show a superficial similarity to *Lepocinclis*, the position is quite different: the cell body itself is very soft but is encased in a rigid envelope composed of other material. This is mainly ferric hydroxide impregnated with a brown manganic compound. The cell body very often adheres to it only with a part of its surface.

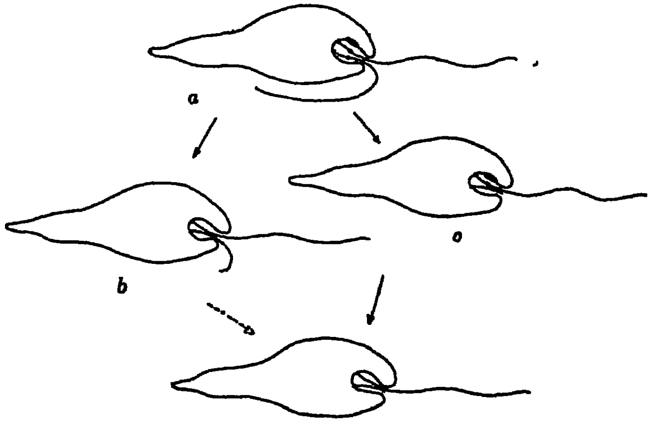

Fig. 4. Hypothetical evolutionary relationship of *a*, *Eutreptia*; *b*, *Distigma*; *c*, *Euglena*; *d*, *Astasia*. *b* may have been developed from *a* by the loss of the stigma and the flagellar thickening (*Eutreptia* spp. with a shorter, and *Distigma* spp. with a longer, second flagellum have been recorded). *c* may have developed from *a* by the second flagellum becoming non-motile and losing its thickening. *d* has developed from *c* by the loss of the stigma and the flagellar thickening. *d* may also have developed from *b* by further shortening of the second flagellum.

The periplast of the Euglenineae is more or less spirally striated but the distinctness and the spacing of the striation vary greatly from species to species and are not quite constant within them. Where this ornamentation cannot be observed in the unstained, living organism, colloidal stains make it readily visible. Neither the distinctness nor the spacing of the striae seem to be related to the ability to undergo changes in shape described as euglenoid metaboly. These changes are probably due to forces similar to those producing amoeboidy—a comparison which does not, of course, provide any clue to the mechanism by which they are produced. The spiral structure of the periplast seems only to impose certain restrictions on the alterations in form.

Pronounced striae are usually found only in rigid periplasts, for example, in *Euglena tripteris*, *Phacus pleuronectes*, *P. longicauda*, *Lepocinclis ovum*, *Rhabdomonas costata*, *R. incurva*, but they may be quite distinct in metabolic species, for example, in *Euglena granulata*, *Astasia curvata* and *A. inflata*. Recently an extremely metabolic new *Distigma* was found, close to *D. proteus* in appearance, but showing marked striation even under low power magnification. This form will be described as *D. striata* n.sp.

On the other hand, there are Euglenineae which appear smooth if not specially stained, not only among the metabolic forms (for example, *Euglena mutabilis*, *E. gracilis*, *E. deses*, *Astasia clava*, *A. parva*, *Distigma Sennii*) but also among more or less rigid species (for example, *Menoidium cultellus*, *Euglena acus*, *Astasia linealis*). The failure to correlate metaboly and striation argues against any hypothesis which assumes a connexion between euglenoid movement and the presence of contractile fibres in the periplast or a skeletal function of the striae.

Euglenoid metaboly is difficult to describe in a few words. The phenomenon is complex and assumes manifold forms. As a first attempt at simplification two types may be distinguished. (1) In *Euglena acus* and *Astasia linealis*, as long as the cells are healthy, nothing more than slight curvature of the body occurs. Only in moribund cells do abnormal distortions and swellings take place. In other species, for instance in *Euglena anabaena*, *E. granulata*, *E. viridis*, this curvature is much more pronounced and leads to the formation of sack-like bulges on the concave side. For the rest, alterations in form in these species are restricted to elongation with a decrease, and contraction with an increase, in width of the middle of the cell. (2) While the alterations so far described do not involve marked local changes in the extensibility of the periplast, this seems to be necessary in the second type of metaboly. In a manner, reminiscent of the bulging of the surface prior to pseudopodia formation in amoebae, certain parts of the periplast yield under internal pressure so that processes arise at unpredictable places. In the less extreme types of such metabolic alterations, certain parts of the cell body swell and shorten to form more or less spherical inflations while others shrink and become attenuated. Both phenomena are strikingly exhibited by *Eutreptia viridis* and *Distigma proteus*. Other species of *Distigma*, *Euglena mutabilis*, *Astasia Klebsii*, *A. Dangeardii*, and the majority of this genus show only this latter type of metaboly. The type and degree of metaboly are characteristic features useful in taxonomic differentiation.

(3) *Pigmented and colourless forms*

In the Euglenineae, as in the Chrysophyceae, Cryptomonadineae, Chloromonadineae and Dinophyceae, there are to be found all the types of nutrition which exist among non-bacterial lower organisms: photo-autotrophy (holophytism), mixotrophy, saprotrophy, zootrophy (holozoism), and parasitism; the last three are often associated with the absence of chlorophyll. Such colourless organisms require organic food, either in the form of dissolved substances (saprotrophy) or as food particles (zootrophy).

No pigmented euglenoid organism ingests food particles, nor does any pigmented cryptomonad or chloromonad, although many pigmented chrysophycean and dinophycean species do so. In the absence of such ambitrophic forms we have no clue as to the changes that have taken place in the evolution of holozoic from holophytic Euglenineae. Some of the zootrophic Euglenineae show structural features obviously adapted to their mode of nutrition, for instance, the so-called siphon or 'Staborgan' in the genera *Peranema*, *Entosiphon* and *Urceolus*; while others (*Heteronema*, *Anisonema*, *Euglenopsis*) do not differ strikingly in their morphology from the saprotrophic genera. The latter are only to be distinguished from the green forms by the lack of chromatophores, *Astasia* being parallel to *Euglena*, and *Distigma* to *Eutreptia*; while there are no green genera corresponding to the closely interrelated *Menoidium* and *Rhabdomonas*, nor a colourless genus, to *Lepocinclis*.*

Without experiments in culture it cannot be established whether a peculiar green species can or cannot multiply photo-autotrophically. This has been demonstrated in a few cases (*Euglena gracilis*, *E. viridis*), but the majority of species are believed to need supplementary organic nitrogen compounds (*E. deses*, *E. agilis*); or their growth is at least so much favoured by these that a mixotrophic nutrition is obviously to their ecological advantage. Experimental results apply only to the particular strains that have been cultivated, so that generalization is not as yet possible.

Only a small number of green euglenoid species have so far been grown in the dark, namely, *E. gracilis* (Zumstein, 1900), *E. Mesnilii* (Lwoff & Dusi, 1935) and *E. cyclopicola* (own observations). All three cease to produce chlorophyll in the dark and therefore become yellow from the carotenes still retained in the plastids. In contrast, all the Volvocales so far grown in the dark keep their green colour; in this respect, as in others, they are nearer to the Chlorococcales than to the rest of the Flagellata. In my experience, Chrysophyceae behave in the dark like the Euglenineae.

Pigmented species of the Euglenineae, though apochlorotic in the dark, produce chlorophyll in their plastids as soon as they are exposed to the light, while truly colourless species do not contain plastids and so remain colourless under all circumstances, not even becoming yellow. They must therefore have originated from green ancestors by apoplastidy, that is, by the loss of their plastidome (cf. also Hollande, 1942, p. 141).

III. TAXONOMIC CONCLUSIONS

The three types of nutrition: phototrophy, saprotrophy and zootrophy, are to some degree reflected in the organization of the different forms. Parasitism may be associated with all three types of nutrition. It is, however, difficult to fix taxonomic boundaries between these groups, as Fritsch (1935, p. 741) and Jahn (1946, p. 265) have already pointed out. For practical purposes, the green species may be separated as Euglenaceae from the saprotrophic Astasiaceae and the zootrophic Peranemaceae. These divisions do not, however, seem to coincide with true taxonomic groups.

The main difficulties are as follows. Certain colourless saprotrophic species are obviously nearer to certain green *Euglenae* than to the rest of the Astasiaceae. This is the case in *Astasia linealis* Pringsheim (syn. *Cyclidiopsis acus* Korshik.), and even more so in the colourless variety *Euglena acus hyalina*, both belonging to the same collective species *E. acus*, comprising *E. acutissima*, *E. rigida*, *E. limnophila* and others (Klebs, 1883; Skuja, 1939), while neither bears a close resemblance to, for example, *Astasia Klebsii*, *A. Dangeardii* or the other members of this genus. The difficulty in defining the line of demarcation between the Euglenaceae and the Astasiaceae is yet more evident in colourless forms of *Trachelomonas* and in apochlorotic species resembling *Phacus* to which I gave the generic name *Hyalophacus* (Klebs, 1883; Pringsheim, 1936; Pochmann, 1942). Varieties of the latter, with and without an eye-spot, have been recorded, similar in shape to *Phacus pleuronectes*. Because of this resemblance such forms have never been referred to *Astasia*.

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* With the possible exception of Gyropaigne, Skuja (1939, p. 113).

Where distinctive features are lacking, as in most colourless Euglenineae without or with an eye-spot, for instance those of the *Astasia quartana* group, the relation to individual green forms is difficult to trace, the more so as the chromatophores and pyrenoids are lacking which, to my mind, are such valuable indicators of natural relationships. Of the several colourless, and therefore *Astasia*-like, species with an eye-spot (Pringsheim, 1942), most are not as metabolic as *Astasiae* generally are and appear to be nearest to *Euglena gracilis*. One of the strains kept in culture, however, is markedly more metabolic, while another is characterized by its posterior muciferous corpuscles (corps mucifères)—features which possibly indicate taxonomic differences. *Astasia longa*, still nearer to *Euglena gracilis*, differs only from the latter in its lack of chromatophores and eye-spot.

The creation by Jahn & Kibben (1937) of the genus *Khawkinea* for the colourless *Euglenae* with an eye-spot, though at the first sight plausible, does not solve the problem of their taxonomic position. *Khawkinea linealis* Jahn (syn. *Astasia linealis* Pringsheim) is more closely related to *Euglena acus* (Pringsheim, 1942, p. 189) than to *Khawkinea ocellata* Jahn & Kibben and *K. quartana* Jahn (syn. *Astasia quartana* Pringsheim). Moreover, the apochlorotic varieties of the *Euglena acus* and the *Phacus pleuronectes* group cannot be allotted to different genera according to the presence or absence of eye-spots, any more than this could be used as a character for generic differentiation in *Polytomella*, *Chlamydomonas*, *Polytoma*, *Hyalogonium*, *Cryptomonas* and *Anthophysa*.

Hypothetical schemes of phylogenetic relationship such as may be found in some of Pascher's papers, in which recent biological forms are arranged in sequence to show successive changes in ancestral forms, are rather out of fashion at present, but Hollande (1942, p. 162) has revived this procedure for the Euglenineae, by beginning with a form possessing chromatophores and an eye-spot, followed by one with leucoplasts and eye-spot, next by one with an eye-spot but devoid of plastids and so forth. Apart from some of these steps being very doubtful (for example, that with leucoplasts), this is not the way things have happened. Ternetz (1913) and Sauer (1935) have seen *Euglena gracilis* transformed into forms like *Astasia* without an eye-spot in a single step, and I can confirm these observations.

New features of taxonomic value may be found in the future but, until they are, the best course seems to be to place all saprotrophic forms not closely related to green species in *Astasia*, which is then still no more artificial a genus than *Euglena*. If a colourless form exhibits features betraying its ancestry, as, for instance, the shape of *Hyalophacus* or the envelope of *Trachelomonas reticulata*, it should be placed near its pigmented relations, as is done with colourless diatoms and Peridineae.

Zootrophy seems to have developed at least twice among the Euglenineae. This would mean that the Peranemideae are phylogenetically independent of the Euglenopsideae—as I would like to call the other subfamily; but the paucity of our knowledge precludes any extended discussion of the phylogeny of the group. Fritsch's (1935, p. 741) proposed separation of zootrophic forms with siphons from those without accords well with the facts, but the amalgamation of the latter with the Astasiaceae, that is the saprotrophic Euglenineae, is not quite satisfactory, because none of the closely related green forms shows any trace of zootrophic nutrition.

Taxonomic interrelations among the green euglenoid genera are rather confusing. If it is justifiable to regard the nature of the plastidome as one of the main taxonomic characters, then the species of *Lepocinclis* and *Phacus* are nearer to *Euglena acus* and *E. tripteris* (cf. Pochmann, 1942, p. 215) than are the latter to *E. viridis* and *E. granulata*, and the species of *Trachelomonas* are nearer to *E. gracilis* and *E. anabaena* than are the latter to, for instance, *E. spirogyra* and *E. splendens*.

The objection that in making such statements too much emphasis is laid on the appearance of the chromatophores may be met by the following considerations. The similarity between *Lepocinclis* and *Phacus* and certain species of *Euglena* is based not only on the plastidome, but also on the rigidity and sculpturing of the periplast and the existence of characteristic, conspicuous paramylon bodies. *Phacus splendens* (Pochmann, 1942) differs from the rest of the genus in possessing pyrenoids and only two chromatophores and a cell body not flat but broadly elliptical in cross-section. It is better placed in *Euglena*. Again, the similarity between certain species of *Euglena* and the naked cells of *Trachelomonas* is based not only on the shape of the chromatophores and the existence of double-sheathed pyrenoids, but also on the configuration and the marked and striking metaboly of the cell body.

The splitting up of *Trachelomonas* into hundreds of taxonomic forms is not comparable to the differentiation of *Euglena* into species. The envelope of *Trachelomonas* is a dead exudation, the structure of which is readily modified by environmental influences as observation in nature and culture experiments have shown. If these exudations were not produced—as may happen under certain artificial conditions—the number of *Trachelomonas* species would be much lower. The shape and the sculpturing of a *Euglena* cell, on the other hand, and still more the inner structure of the protoplast and its organs, are the expression of specific inherent properties of its protoplasm, not readily modified by external conditions. They should be credited with deeper significance as taxonomic features than the variations in structure of *Trachelomonas* envelopes.

The taxonomic conclusions reached by these considerations are still insufficient for it to be possible to reconcile theoretical leanings towards a natural grouping with the practical need for establishing a really workable system for the Euglenineae; nor will they, I am afraid, facilitate the creation of better identification tables. They may, however, be of use in directing the attention of future observers to the taxonomical features most likely to reflect natural relationships between the various groups.

IV. SUMMARY

The Euglenineae form a well-defined natural group comprising a great number of species, the subdivision of which into families is difficult to make.

Among taxonomic features, the basic shape of the cell body is fusiform but often irregular and twisted, with periodical tapering towards the posterior end. It may be concealed by morphological aberrations. The metaboly of many species and the occurrence of dimensional varieties render the use of shape and size as specific features sometimes rather equivocal. Other features have to supplement them for the identification of the species.

The shape of the nucleus is sometimes characteristic, but the structure of the plastidome and the presence or absence of pyrenoids are of much greater taxonomic importance. These features vary greatly in the species of *Euglena*, while the other green genera have almost uniform chromatophores. By plastid and other characteristics *Trachelomonas* and *Colacium* are related to certain species of *Euglena*, while *Phacus* and *Lepocinclis* are nearer to others.

The flagellar apparatus of the Euglenineae is tentatively considered as being composed of two flagellar units throughout the group, the length alone varying according to the genus. *Eutreptia* with two equal or more or less unequal flagella would be nearest to the hypothetical ancestral form. The other green genera would be derived from it by a further shortening of the minor flagellum. Its near convergence to the long active flagellum gives the impression of bifurcation.

Distigma would be the apoplastidic counterpart of *Eutreptia*, *Astasia* that of *Euglena*. All green Euglenineae and some colourless ones have an eye-spot and opposite to it a thickening of the active flagellum or flagella not found in the species without an eye-spot.

The striation of the periplast varies greatly, without correlation to the degree of cell metaboly. A first attempt at classifying the different types of metaboly is made. Envelopes differ from the periplast by being exudations of inorganic substances.

While the distinction between the phototrophic and the saprotrophic Euglenineae seems gradually to disappear, the holozoic forms are more distantly derived. There are no indications as to their evolution, as no zootrophic green Euglenineae are known, and some are similar to saprotrophic forms. The entire class of the Euglenineae is rather uniform, and a rational and at the same time natural classification has not yet been attained.

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THE DISTRIBUTION AND BIOLOGY OF HAKE

By T. JOHN HART
'*Discovery*' *Investigations, 52 Queen Anne's Chambers, London, S.W.* 1

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I. INTRODUCTION

The distribution and biology of the several closely allied species of hake (*Merluccius*) is of peculiar interest because of their wide geographical range, their predominance in fish faunas over very large areas and their great economic importance. A group presenting these features may give good evidence of the relation between animal and its environment, illustrating many of the main trends in the ecology of demersal marine fishes. Further study along these lines may assist in the elucidation of the evolutionary history of the group, which would obviously have some bearing on wider problems of evolutionary theory. Finally, since at least three species of hake are already intensively exploited by man, the group may provide exceptionally good illustrations of the effect of fishing on the stock, a most important aspect of economic marine biology. This has been very well worked out for the European species by Hickling, as will presently appear, but further studies of the lesser known species on similar lines are still needed.

A great deal of work on hake has been done since Belloc (1929) published his monograph on the genus. The taxonomy has been revised by Norman (1937), and enormous advances in our knowledge of the general biology of the European species have resulted from the work of Hickling (1927, 1928, 1930, 1933, 1935, 1946). Observations on Patagonian hake, made during the trawling surveys organized by the *Discovery* Committee, which would normally have been written up by the late E. R. Gunther, have also recently become available (Hart, 1946). As a result of these works and others, Belloc's monograph is out of date, though it contains much that will aid students of the group for many years to come. Yet current general books on fishes, and even Norman (1937) in his systematic revision, still refer the reader to Belloc for a general account of the genus, though this may be unavoidably misleading in view of the subsequent work.

A new monograph of the genus is needed, but the time is not altogether propitious for attempting one, because the rapidly increasing commercial importance of at least two of the less known species makes it practically certain that detailed researches upon them must be begun soon. Hence a monograph of the genus written within the next few years might be out-dated even more rapidly than was Belloc's.

The aim of this review is to collate the more recent data concerning the distribution of the several species of hake—one of the preliminaries necessary before any future synthesis can be attempted—and to demonstrate the great interest of the group from the point of view of general biology.

II. POSITION OF THE GENUS *MERLUCCIUS* RAFINESQUE

Most recent writers on the taxonomy of the Anacanthini follow Gill (1884) in placing the true hakes in a family Merlucciidae, separate from Gadidae, with which they were formerly classified, and Macruridae. There is still room for doubt as to the propriety of this arrangement, for the osteological characters upon which the distinction is based are shown equally strongly, though not in combination, by various genera of Gadidae. For example, the elongated otoliths of the hake (a point stressed by Belloc) are surpassed in length by the (admittedly more massive) otoliths of *Urophycis*. The one real advantage of this rather artificial revision in taxonomy seems to be the expression it gives to the close relation between the true hakes and *Macruronus*, a Patagonian and New Zealand genus formerly classified with the Macruridae because it lacks a separate caudal fin. Norman (1937) has shown that in cranial characters *Macruronus* comes nearer to the true hakes than any other known Anacanthini, and placed it in the family Merlucciidae. The shallow depths frequented by *Macruronus* also indicate their wide divergence from Macruridae which are, almost exclusively, inhabitants of deep water.

The genus *Merluccius*, which alone concerns us here, is itself well defined; the absence of a barbel is a good superficial distinction from the nearest related gadid genera, such as *Urophycis* (called hake in America). I have used the word hake in its Middle English (and current English) sense, for *Merluccius*. In America 'hake', with or without various prefixes, has been applied to several species of *Urophycis*, although there is a 'true' hake, *Merluccius bilinearis*, locally termed 'silver-hake' or 'whiting', on the same coasts. There seems little justification for the American usage, since hake meant *Merluccius* before America was settled by Europeans. Although dictionaries give the etymology of the word as doubtful, its affinity with the German 'Hecht'—pike—seems clear. Thus 'See-Hecht', modern German for hake, has the same literal meaning as the Latin-French 'Marlutius' or *Merluccius*. A common Germanic (? Saxon) origin for our 'hake' and the German 'Hecht' seems probable. The Anglo-Saxon 'hacod'—hooked—may be involved, since prior to trawling almost all hake caught were hooked.

III. GENERAL BIOLOGY

For proper appraisal of the distributional features about to be discussed some idea of the general biology of the group is needed, and the following brief summary, taken mainly from the works of Hickling (quoted above), Bigelow & Welsh (1925) and Hart (1946), is an attempt to provide this background.

The true hakes are slender soft-rayed bony fish of medium or large size inhabiting warm temperate and subtropical seas. They are more abundant, and the individuals attain a larger size, nearer to the polar limits of their range than the equatorial limits. This effect is an example of the 'polarity' displayed by many marine animals (Wimpenny, 1941). It is very well shown by the Patagonian hake, and even more definitely by the neighbouring and relative fish *Macruronus magellanicus* (Hart, 1946). It is connected with the temperature, though probably not very directly. While one may say that optimum conditions for the several species of hake seem to be found nearer to the coldest waters inhabited by them than to the warmest, it is almost certain that many factors other than the direct effect of temperature combine to produce this result.

The growth rate of hake is moderate and the females take much longer than the males to reach sexual maturity. Hickling (1933) has shown that female European hake increase in length by annual increments of some 8–9 cm. over the period from the second to the eighth year. They are just over 20 cm. long at the end of the second year of life, so that earlier growth is somewhat faster, and after the eighth year the growth rate diminishes as in so many other fishes; but over the main growing period the relation between length and age is almost linear, instead of the familiar continuously diminishing curve. Hickling found that otoliths give a more reliable guide to age in this genus than scale readings such as were used by Belloc. Otoliths are not always satisfactory, but Hickling's exhaustive biometric data show that over 60% of them were legible, and gave readings reasonably consistent with length distributions.

European hake commonly exceed 1 m. in length if they survive to their thirteenth year. The South African species may also attain this size, which is approached (as an extreme) by the other members of the genus, though they are probably all somewhat smaller on the average. In European hake the females commonly reach larger sizes than the males, which grow more slowly after maturing in their third year of life, while the females commonly attain 8 years before first spawning. The discrepancy in size between the sexes is even more marked in Patagonian hake (Hart, 1946).

European hake spawn late in spring or in summer, when the fish are moving shorewards into comparatively shallow water. The older, larger individuals seem to take the lead in this annual migration, and spawn earlier in the year than the smaller mature fish. The first part of the seasonal shoreward movement, characteristic of so many diverse fishes, is thus a breeding migration, and during it these normally voracious animals feed less greedily than at other times; but the same shoreward trend may continue after spawning, when the spent fish are recuperating rapidly at the expense of mackerel, clupeoids and other shoaling fishes. Moreover, the immature individuals show similar seasonal movement, though later and lesser in extent, for they do not proceed so far offshore as the larger fish on the converse outward migration that begins in autumn. It is a most interesting fact that immature hake show seasonal variation in condition parallel to that of the spawning fish, though less pronounced, as one would expect. Except in the earlier half of the rather prolonged spawning season, when relatively large fish may be concentrated in comparatively shallow water, the movements of the hake of all ages result in a distribution in relation to depth of water similar to that common to so many other demersal fishes—the larger fish are found at the greater depths.

Hake, which are nocturnal mid-water feeders, demersal only by day, proceed farther offshore in winter than most of the fishes that undertake similar migrations. In winter their range extends beyond the edge of the continental shelf, over oceanic depths. In general they are found at greater depths than other species commonly trawled for, as is instanced by Barnard's (1925) record that Macruridae are prominent in the diet of the South African species. When offshore, the European hake feed very largely upon the blue whiting, *Micromezistius poutassou*, as Hickling (1927) has shown. It is a curious fact (Hart, 1946) that although a close relative of the blue whiting, namely, *M. australis*, is present off the Patagonian continental shelf, the Patagonian hake have not yet been observed to feed upon it, although some hundreds of stomachs have been examined. In their general onshore and offshore movements, however, the Patagonian hake show essentially the same behaviour as the European species (allowing for the reversal of the seasons). The same may be said of the silver hake or 'whiting' (*Merluccius bilinearis*) of New England (Bigelow & Welsh, 1925), and such scanty evidence as we possess for the other, less known species, strongly suggests similarity of behaviour in this respect among all members of the genus.

While sizeable hake are mainly ichthyophagous, the smaller, younger fishes feed largely upon krill—various species of shoaling Euphausiidae—and other macroplanktonic Crustacea such as hyperid Amphipoda and post-larval *Munida*. Larval hake appear to feed upon nauplii and small calanoid copepods soon after hatching, and eat larger calanoids before reaching a length of 1 cm. (Lebour, 1920). The marked change in diet seems to occur at about the age when the males reach maturity (third year of life in the European species; Hickling, 1933). However, the females also begin to eat more fish at about this age and size, although they do not mature until very much later. Thus the marked change in feeding habits at a certain age is not so closely linked with the onset of sexual maturity as it seems to be in so many other fishes of widely diverse habit (e.g. *Macruronus*; Hart, 1946). It is very interesting to note that an essentially similar change in feeding habits has been shown even in fresh-water fishes by Hartley (1945).

The growth and timing of the seasonal movements of species covering such a wide range of latitude as the true hakes naturally show considerable local variation. Thus in the European species the Mediterranean stocks—racially though probably not subspecifically distinct—never attain the sizes shown among the stocks of the Atlantic seaboard; and near the southern limits of the species' range off the north-west coast of Africa, the onshore movement takes place much earlier in the year than it does farther north.

IV. IDENTIFICATION OF THE SPECIES

Norman (1937) recognized seven species of true hakes:

Merluccius merluccius (Linnaeus)	Europe, N. Africa	15
M. hubbsi Marini	East coast of Patagonia	Numerous
M. productus (Ayres)	North Pacific	5
M. gayi (Guichenot)	Pacific coast of S. America	4
M. bilinearis (Mitchill)	Atlantic coast of N. America	8
M. capensis Castelnau	South Africa	17
M. australis (Hutton)	New Zealand	3

The figures denote the number of specimens that Norman examined.

Belloc (1929) recognized five species only, lumping *M. hubbsi*, *M. gayi* and *M. australis* together as *M. gayi*, but excepting the European and South African species he was working entirely on second-hand evidence, and many authors had long regarded *M. gayi* and *M. australis* as identical. Belloc was unaware of the existence of hake at least as far north as the mouth of the River Plate on the east of South America, although they had been exploited, on a small scale, for nearly 20 years (Devincenzi (1926), who also confused *M. hubbsi* with *M. gayi*). These facts combine to render erroneous Belloc's account of the distribution of the species, although he clearly perceived some of the main features of the distribution of the group as a whole.

The species of hake are all very closely allied, and apart from the confusion of three of the southern species, we find good authorities expressing doubt as to the distinction between the European and South African forms (Barnard, 1925; Hickling, 1927). There is also a remote possibility that the very dark individuals reported near the southern limits of *M. merluccius* off the west coast of Africa, and believed to be but a race of that species (Belloc, 1937), may prove to be an eighth, hitherto unrecognized tropical species, bridging the gap between *M. merluccius* and *M. capensis*. I think this unlikely, but specimens recently collected by Dr Bruun on the Danish *Atlantide* expedition may throw new light on the subject.

Belloc (1929) is almost certainly right in regarding *M. argentatus* Faber, of Iceland, as invalid. Saemundsson (1927) declared that it was evidently a large whiting, and the fin-ray formula could be made to fit this view. But Saemundsson himself had shown that *M. merluccius* (rarely) straggles as far as Iceland, and Belloc's own suggestion, that it was an abnormal (? albino) individual of that species, seems more probable. Since first reported by Garstang (1900) it has been found that albinism in hake, though rare, may occur over a wide area.

The problematic specimens of *Merluccius* obtained by the *Albatross* at four deep-water stations in Panama Bay, and described by Garman (1899) as *M. angustimanus*, may constitute another species. They seem comparable to the problematic specimens from West Africa south of the normal range of *M. merluccius* but thought to be but a race of that species. Norman (1937) concluded that *M. angustimanus* was most probably a form of the southern species *M. gayi*, already known to range northwards nearly to the equator off the Peruvian coast. The *Albatross* specimens were obtained in about 7½° N., and I can trace no further records of the species. Norman's and Garman's accounts show it to be nearer to *M. gayi* than to the north Pacific species, *M. productus*. Hildebrand (1946) is apparently inclined to agree with Norman's view, but he refers to *M. angusticeps* when speaking of the *Albatross* specimens (? in error).

Norman's distinctions seem sound, but the number of specimens of the less accessible species that he was able to examine was very small, and I think he would have been the first to agree that further work on the subject—on a biometric scale—is needed. A 'skeleton key' based on Norman's (1937), but shorn of overlapping characters, so that each species has one unequivocal distinction from any of the others, is shown here. Four characters are needed to delimit the seven species:

(1) Relative size of scales determined by counts of a longitudinal series below the lateral line.

(2) Number of gill-rakers in the lower part of the anterior arch.

(3) Degree to which the pectoral fin extends posteriorly in relation to the origin of the anal fin.

(4) Relation of depth of body to body-length.

These characters are known to be subject to considerable variation, and pending the collection of large amounts of numerical data the limits of their variation remain in doubt. Moreover, nos. (1) and (4) are very difficult to determine with any precision in practice, and even nos. (2) and (3) as used by Norman are not wholly satisfactory, as I hope to show elsewhere. Thus while it is probable in view of the discontinuity of the known geographical distribution of hake that further work will vindicate the distinctions recognized by Norman, the need for continued investigation of them remains clear.

Artificial key to the species of the genus Merluccius *based on that of Norman* (1937) *but excluding overlapping characters*

1 (12) Less than 155 scales in a longitudinal series below the lateral line.
2 (5) 115 or fewer scales in a longitudinal series.
3 (4) Pectoral extends beyond fore end of anal. *M. gayi* (Guichenot)
4 (3) Pectoral does not extend beyond fore end of anal. *M. bilinearis* (Mitchill)
5 (2) More than 130 scales in a longitudinal series.
6 (7) Less than 10 gill-rakers on lower part of anterior arch. *M. merluccius* (Linnaeus)
7 (6) 10 or more gill-rakers on lower part of anterior arch.
8 (9) Pectoral not extending to origin of anal. *M. hubbsi* Marini
9 (8) Pectoral extends to or beyond origin of anal.
10 (11) Depth of body 7 or more in the length. *M. productus* (Ayres)
11 (10) Depth of body considerably less than 7 in the length. *M. capensis* Castelnau
12 (1) 155 or more scales in a longitudinal series below the lateral line.
M. australis (Hutton)

The very difficulty which we experience in finding distinction between the species of hake points to their close relationship, and to the probability that their present localization may be a comparatively recent development. The study of their distribution is thus of outstanding interest. How did this distribution arise? Which hake are most primitive? In what centre did a group, dominant in fish faunas over such wide sea areas to-day, have its origin? Such questions can only be answered hypothetically, if at all, in the present state of our knowledge, but their bearing on larger problems of the evolution of the Anacanthini is obvious. It is also obvious that two prime requisites for their solution are further development of systematic work on the basis of Norman's revision, and further amplification of our knowledge of the distribution of hake.

V. ECONOMIC IMPORTANCE

For a brief consideration of the economic importance of the several species of *Merluccius*, we may take them in order of the degree to which they have been, and are, exploited by man.

M. merluccius, the *merluche* of heraldry, has been an important constituent of the fish food of the western European nations throughout historic times. In Britain hake-fishing rights have been the subject of foreign commercial treaties, perhaps in pre-Norman times and certainly from the time of King John to the reign of Queen Mary. Much hake was eaten during Lent. Latterly it fell into disfavour, partly perhaps because the disestablishment of the Church led to a less rigorous insistence upon traditional lenten fare, but chiefly because improved boats and gear enabled fishermen to catch far greater quantities of the choicer fish than before. In France, Spain and Portugal the hake must always have been relatively important, owing to lack of the colder water gadoids near at hand. This would apply even more forcibly in the Mediterranean, where hake have been mentioned in scientific literature from the time of Aristotle (Couch, 1864; Day, 1880–4). It is an interesting fact that Faber (1883), who shows that hake was the principal fish captured at Fiume in 1879–80, also mentions that the superiority of hooked over net-caught hake was so marked that they could command an appreciably higher price. In Britain, however, during the latter half of the last century, we find the learned forefathers of British ichthyology dismissing it as a poor coarse fish of inferior table qualities. It is said, indeed, that the German and Dutch names for hake, 'Stockfische' or 'Stokvische' (there are others), derive from the custom of letting smack's boys keep them for 'stocker' of personal perquisites, which makes it certain that they were practically unsaleable.

The rapid development of otter trawling, especially steam trawling, at the turn of the century, saw a rapid decline in the *proportion* of prime fish landed (though of course the actual quantity was at first greatly increased) and the development of fried-fish shops greatly stimulated hake trawling. Indeed, Hickling (1935) has been able to show that there was serious depletion of the stock through over-fishing before the war of 1914–18. During that war all fish stocks recovered to some extent, and with improved boats and gear and the development of deep-sea trawling on distant grounds throughout the whole latitudinal range of the species, British hake trawling showed increasingly heavy catches until 1925. But the catch was only maintained by increased fishing effort, as Hickling (1935) has so clearly demonstrated. From 1925 the catch declined and depletion continued until 1939. War again enabled the stocks to increase—Hickling (1946) has shown how rapidly the catch per unit of fishing effort rose after 1941—but now, although the fishing fleet has by no means regained its pre-war strength, the danger of depletion is again imminent.

The importance of hake to the modern British trawling industry is very great. It is the staple catch of our great west-coast trawling ports, Fleetwood, Milford, Cardiff and Swansea. Even in 1930, when decline of the stock was beginning to show itself, hake ranked third of all our trawled fish, whether reckoned by quantity or by value. Only cod and haddock were more important. Between 1920 and 1925 the British landings of hake averaged 38,500 tons annually, and some of the fleet were working as far south as North Africa in winter. Throughout the inter-war period the intensity of fishing by French and (latterly) modern Spanish vessels, in the southern part of the range of the species, also greatly increased. In France the great hake port is La Rochelle.

Though the price of hake is influenced by many economic factors besides supply, the average value of the British catch at first sale for the period 1920–33 was £1,532,000 per annum (Hickling, 1935). More recent figures are given in the monthly returns issued by the Ministry of Agriculture and Fisheries, and some of their implications are dealt with by Hickling (1946). It seems clear that the future of the industry depends on acceptance of some degree of restriction of output, otherwise over-fishing, with gradual depletion of the stocks, is bound to recur.

The history of the commercial exploitation of *M. bilinearis* affords a close parallel to that of the European hake, but with a characteristic acceleration due to the American fishermen having a totally undepleted stock to deal with when modern methods of capture and treatment of the catch were introduced. In dealing with the silver hake ('whiting' of New England) it is important to realize that much of the earlier literature is vitiated by confusion between it and the several species of *Urophycis* (Gadidae) which are called 'hake', with or without distinguishing prefixes, along those coasts. The once important by-product trade in 'hake sounds' (swim-bladders) for isinglass was based on these and not on *Merluccius*.

In the early days silver hake was regarded as rubbish by the New England fishermen, and also as a great nuisance when large numbers were caught in mackerel nets (Goode *et al.* 1884) and had to be discarded. Their inshore migration in summer is even more marked than that of the European species, and they frequently become stranded in pursuit of prey. At times they were used for manure (Bigelow & Welsh, 1925), and as recently as 1895 only 37,000 lb. were marketed from Massachusetts and Maine. By 1919 more than 14 million lb. were sold, and even at that date most were still caught in traps and weirs; the price was too low for the offshore fishermen to bring in those they caught. Since then the demand has risen enormously with development of large cold stores, especially in the mid-western States. Fiedler (1943) shows that nearly 50 million lb. of *M. bilinearis* were marketed in 1940, more than 80% of the total coming from the New England States. Nearly half this catch was frozen (11% of the total frozen fishery produce of the country). It had, indeed, become the most important single species of the frozen fishery trade. This rapid development synchronized with a big change in fishing methods, more being taken in otter trawls than in pound nets now, except in New Jersey and Rhode Island. Figures from the American official statistics show that between 1929 and 1940, while the total landings of fishery produce increased by some 50–75% (allowing for annual fluctuations), the *Merluccius* landings increased threefold, and the proportion of *Merluccius* in total landings was more than doubled. One wonders how long it will be before the over-fishing problem becomes manifest with the New England 'whiting'.

M. capensis, which is known in South Africa as 'stockfish' or 'stokvisch', has been the staple of the modern trawling industry developed in that country. There are no statistics of the earlier commercial landings to enable one to trace the growth of the modern fishery at the Cape, but the figures for 1929–32 (when the industry was well established) published by von Bonde (1934) leave one in no doubt as to the importance of *Merluccius*. In that period the catch was more than one-third of the fish landed, and averaged some 7850 tons of stockfish, worth over £150,000, per annum.

The Patagonian hake, *M. hubbsi*, is captured by the small trawling industry operating from the mouth of the River Plate. This originated with Don Pedro Galceran in Montevideo, but the Uruguayan enterprise failed, and the business was then carried on from Buenos Aires (Devincenzi, 1926). Trawling surveys on the Patagonian continental shelf to the southward, between Argentina and the Falkland Islands, carried out in the course of the *Discovery* Investigations,* have shown that *Merluccius* would be the staple of any fishery for demersal species that could be developed there (Hart, 1946).

The hake of the west coast of South America, *M. gayi*, is captured by such small-scale fisheries as can operate throughout most of its great latitudinal range, but the abrupt descent of the sea-floor to oceanic depths off that coast, so that there is (virtually) no shelf, precludes modern large-scale trawling methods.

The New Zealand hake, *M. australis*, is as yet little sought after, supplies of choicer species, more readily accessible to the small-scale local industry, being ample. A few are marketed in Dunedin as 'haddock' (Phillipps, 1921).

M. productus of the Pacific coast of North America is likewise neglected owing to the plenitude of better food fishes in those favoured waters, even though large-scale modern industries operate there. As long ago as 1907 the British Columbia Fisheries Commission reported that it was not inferior to the Atlantic species, but it is little exploited even to-day. Such few 'California hake' as appear in the returns for that State are a seasonal by-product of an inshore trawl fishery for flat-fish.

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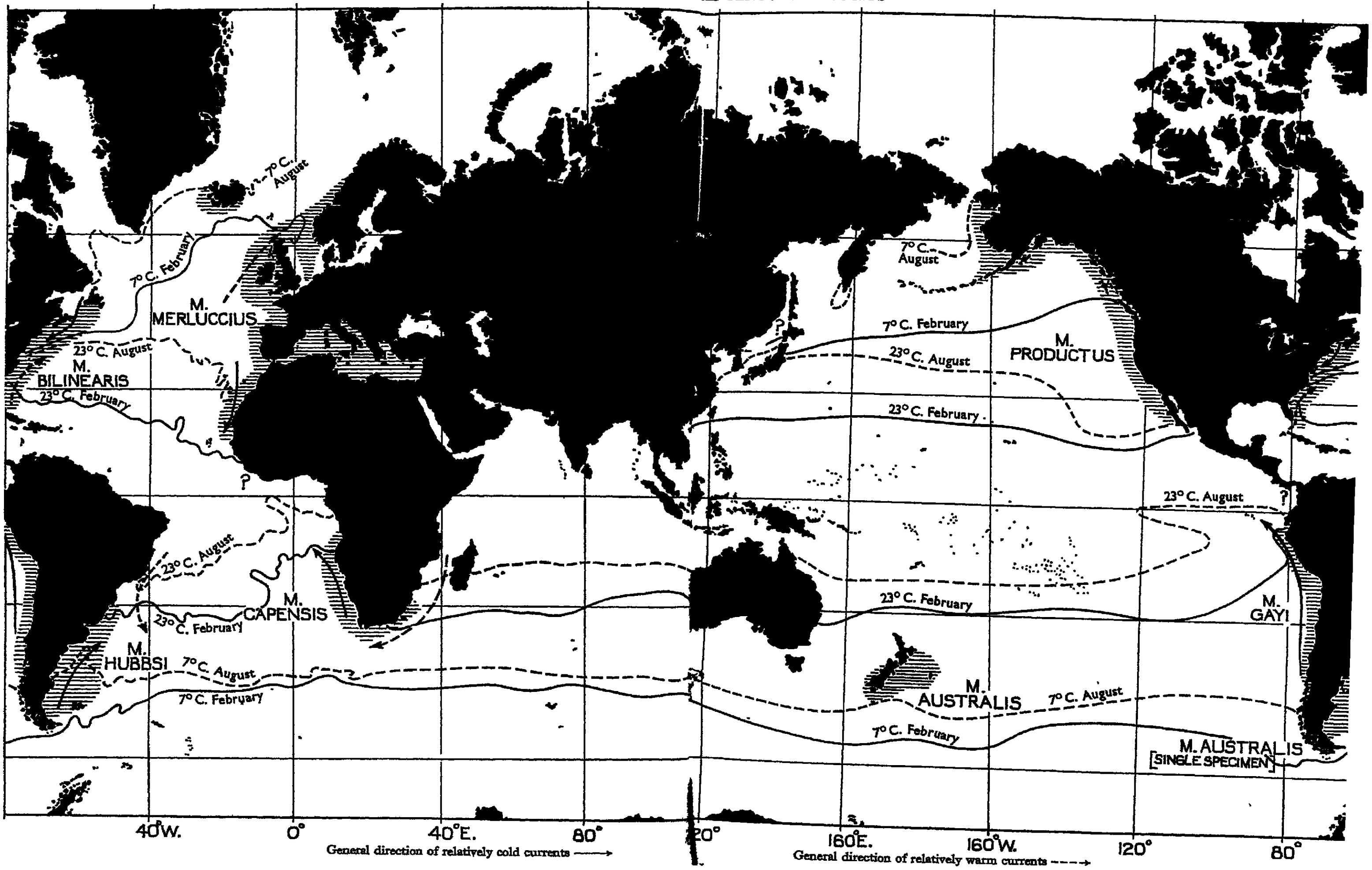
* The late E. R. Gunther was the zoologist most concerned in this work.

VI. DISTRIBUTION

The normal range of the European hake, *M. merluccius*, is from the Norwegian Rinne and Skaggerak (where there seems to be a local race, the 'stumpies' of Hickling, 1930) and from west of Scotland, southwards along the edge of the continental shelf as far as Dakar on the Mauritanian coast, perhaps even farther south. A local race has been reported from Cape Verde (Belloc, 1937). The hake is common in the Mediterranean, especially on the northern shore, but the Mediterranean stock is of small size—probably another local race—with a very different growth rate from that of the more typical stocks along the continental slope off western Europe (Belloc, 1929). Stragglers occur rarely in Icelandic waters (Saemundsson, 1927), but to the north and west of the British Isles it seems that hake are rarely found beyond the isotherm of 8·7° C. (Hickling, 1927, 1928). The regular annual migration of the species into the North Sea (Thompson, 1915) is on a very small scale (Hickling, 1927), and in the southern North Sea it is rare.

Numerous writers have remarked on the disappearance of the population of small hake that used to frequent the western part of the English Channel. Each of the last two wars has been succeeded by a minor recrudescence of these fish. Thus Clark (1923) noted a considerable increase after the war of 1914–18, and through the kindness of Mr F. S. Russell, Director of the Plymouth Laboratory, I have myself been able to observe an increase during the last two years. These Channel hake (or at least those caught on the inshore grounds) are all of small size (12–38 cm.) and from 1 to 4 years old. They are most numerous in summer and autumn, as one would expect from the known migratory habits of the species elsewhere. In the summer of 1927 my former colleague, Mr G. W. Rayner, kept a record of several hauls made on the inshore grounds, under the guidance of Mr E. Ford, and these show that very few hake were to be found there at that time. Examples occurred in about one-third of the hauls, and at no time were they being captured at a rate of more than six fish per hour. Obviously there had been a decrease since the time of which Clark was writing. During the summer and autumn of 1946, on the other hand, baby hake occurred in almost every haul made, and in September more than one score per hour were being taken. Moreover, they continued to occur (in lesser numbers of course) throughout the winter. It seems probable that with the main stock of hake to the westward undepleted 'population pressure' forces a considerable number of baby hake on to perhaps indifferently favourable nursery grounds in the shallower waters of the western Channel. When the breeding stocks are depleted through over-fishing the number of juveniles inshore decreases. I suspect that when one can no longer obtain half a score of baby hake in an hour's trawling in September, on the inshore grounds off Rame Head near Plymouth, it will be almost certain that over-fishing of the main breeding stocks to the westward has taken place. The relative abundance of small hake in this one small intensively studied area, at the time of their greatest shoreward concentration, may prove a valuable pointer to the important changes in the large breeding stocks south of Ireland.

DISTRIBUTION OF THE GENUS MERLUCCIUS



To continue our survey of the wider distribution of the species, we know, mainly from the intensive work of the fishing fleets described by Hickling, that it is the dominant demersal fish along the edge of the continental shelf throughout warm temperate latitudes. It is especially abundant west of Scotland from St Kilda southwards, and along the slope west of Ireland. The latitudinal range, from the Norwegian coast to Dakar, some 45° of latitude, is very great. We have seen that the normal northern limits of the distribution of the species seem closely defined by the 8·7° C. isotherm, and it is probable that the wide range results from the diverging current systems west of Europe and North Africa. To the north we have relatively warm currents trending northwards (positive temperature anomaly in the sense of Böhnecke (1936) and Hentschel (1936), while to the south the relatively cold Canary current flows southwards along the north-west African coast, with a sharp definition from the warmer tropical water, where it swings offshore in about 15° N. latitude. This point, which varies somewhat with the seasons, will I think be found to coincide with the normal southern limits of the distribution of *M. merluccius*. In the Canary current there is, of course, strong negative temperature anomaly. The effect of these two major hydrological features is thus a reduction in the north-south gradient of temperature—a basic factor in the conditions for living—over an exceptionally wide range of latitude. The great latitudinal range of the European hake is, I believe, intimately related to this wide tempering of the conditions, though secondary effects other than the direct influence of temperature are involved.

In a paper read to the Challenger Society (January 1947) Dr Deacon stressed the significance of large-scale water movements in the determination of the distribution of marine animals, and suggested that current boundaries would eventually prove more significant than purely thermal ones in this respect. The point applies with particular force when one is considering the distribution of purely planktonic organisms, but large fishes such as hake are planktonic only for a few months as eggs and larval stages. Nevertheless, these few critical months must render currents the prime factor in determining their main breeding grounds, as Meek (1916) and, later, E. S. Russell (1937), have so clearly stated. At other times, however, it is probably the secondary effect of the greater richness of the relatively cold waters in all forms of macroscopic life, as compared with the tropical waters to the west and south, that determines the extent to which hake penetrate towards the equator. While the actual temperatures (at the depths to which it is known that hake can descend) in tropic regions may be well within the limits they can normally tolerate, the food supply is probably insufficient there.

By 'organic polarity' (Wimpenny, 1941) is meant the sequence of communities, species and physiological groups found in a uniform medium as it stretches from the equator towards either pole. In the sea it is illustrated, in its widest sense, by the less abundant (though more varied) fauna of the tropics as compared with that of the seas of higher latitudes; and in its more restricted sense, by the increasing size of species within groups, or of individuals within a species, towards the polar limits of their latitudinal range. This is well shown within the limits of most groups of marine animals, the larger species being found in colder waters, as *Sagitta gazellae* among chaetognaths, and *Glyptonotus antarcticus* among isopods. Among fishes sharks are an obvious exception, but most teleost groups conform fairly well. Within the limits of single species the positive correlation between increase of size of individuals and increasing latitude (the converse of that observed in many terrestrial forms) is well shown by the copepod *Calanus finmarchicus*, the amphipod *Gammarellus* (*Amathilla*) *homari*, the tunicate *Salpa fusiformis*, and many teleost fishes, including hake.

Towards the polar limits of the range of hake other secondary factors—changes in the nature of the food supply and increasing competition with large colder water gadoids such as cod—combine with the direct effect of the primary factors to form the limiting complex. With regard to the competition factor, it seems highly significant that the South American species of hake ranges southwards into water some 2° C. colder than is to be found near the northern limits of the range of the European species. Off Patagonia large gadoid competitors are absent from the polar limits of the hake grounds, while in north European waters cod and coal-fish become extremely abundant as one proceeds to the northward. It is to be noted that these secondary or derivative factors arising from the primary ones (currents, temperatures, which are themselves interrelated) illustrate in their turn various aspects of the phenomenon of 'organic polarity' which hake seem to show so well in its more restricted form.

In charting the distribution of all the known species of hake, I have used isotherms rather than current boundaries because in many parts of the world the temperatures are the only reliable data yet available. In this I have had valuable assistance from Dr J. N. Carruthers of the Admiralty, who has helped me to adapt some of the data of Schött (1935) and Böhnecke (1936) to the work in hand. I have selected what seemed to me to be the two most critical isotherms, those of 7 and 23° C.,* and given the August and February values for each (Fig. 1). From this it can at once be seen that the distributions of the other species of *Merluccius* conform in a remarkable degree to the thesis we arrived at as a result of the study of the European species; their range seems clearly to be determined by the major hydrological features along the coasts of the continents.

The distribution of *M. bilinearis* and the concomitant hydrological features on the other side of the north Atlantic show the precise converse of the conditions observed in studying the European species. The northern limit of the 'silver hake' is the south shore of Newfoundland, and to the east of Nova Scotia earlier records from the southern side of the Gulf of St Lawrence refer to *Urophycis* spp., not to *Merluccius* at all (Huntsman, quoted by Bigelow & Welsh, 1925). Off the New England coast it is exceedingly abundant, but to the south it is never captured in large numbers beyond North Carolina. Odd examples occur (in deep water only) off Florida and at the Bahamas (Longley & Hildebrand, 1941). Ribeiro's (1915) record of two specimens from the coast of Brazil almost certainly relates to northerly stragglers of *M. hubbsi*, and not to the North American species.

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* Note that this isotherm is the one most nearly coincident with the average position of the Tropical Convergence in the regions where we know it best (Deacon, 1937). The tropical convergence is the boundary between the subtropical and tropical surface waters: two distinct water-masses with markedly different properties.

It is noteworthy that the two true hakes on the Atlantic seaboard of the two Americas, *M. bilinearis* in the north and *M. hubbsi* in the south, resemble one another more closely than they do any other members of the genus. The same may be said of the European and South African species on the opposite side of the ocean, and of the Californian and Chilean species in the Pacific. All three pairs of species show such close superficial similarities as to suggest meridional divergence from common stocks at no very distant epoch. The further problems of the direction in which this can have taken place, or whether it acted in both directions; how the New Zealand species fits into the picture; and where (since all seven species are very closely related) the original stock of a genus so widely dispersed to-day can have been located, can probably be attacked only with the aid of geologists.

The extreme latitudinal range of *M. bilinearis* is thus from 48° N. to 27° N., or 21° of latitude, which is less than half the range shown by *M. merluccius*; and the main hydrological features are a convergence of currents showing temperature anomaly of opposite sign from the diverging currents in corresponding latitudes on the European side of the ocean. The northern part of the habitat of *M. bilinearis* is influenced by cold water from the southern extremity of the Labrador current flowing southwards, and the southern part of its range by warm water of the Gulf Stream flowing north.

In the South Atlantic *M. hubbsi* and *M. capensis* do not show a similar contrast, because the land-mass of Africa does not extend beyond 35° S.; but the influence of main current systems is shown by the distribution of *M. capensis* on the two sides of the African continent, with a clarity equal to that which we have seen in considering the two hake species of the North Atlantic. On the western side of South Africa, where the relatively cold Benguela current sweeps northwards, *M. capensis* is found as far north as Angola. The limit is not precisely known, but it is in about 10° S. On the eastern coast, where the relatively warm current sweeps down from the Mozambique Channel to Cape Agulhas, 'stockfish' are not found north of Natal (*c.* 28° S., Barnard, 1925).

Yet another striking example of relation to major current systems is shown by the distribution of the two hake species on either side of the South American continent. On the Atlantic side the relatively cold Falkland current flows to the north over the Patagonian continental shelf and *M. hubbsi* duly ranges up to southern Brazil. Here there is a sharp convergence with the relatively warm Brazil current that flows southwards down the coast, and usually begins to swing offshore in about 30° S. (varying seasonally). Though occasional stragglers of the species may be found farther north (Ribeiro, 1915), this is the normal northern limit of the species. To the south, where it is dominant in the fish fauna and large competitors are few, it is found as far as Magellan Straits (53° S.) in water considerably colder than that which other hake species normally tolerate (Hart, 1946). The normal extreme range for *M. hubbsi* is thus some 25° of latitude.

On the Pacific coast of South America *M. gayi* extends from southern Chile to Paita in Peru (4° S., Evermann & Radcliffe, 1917). Its range is thus nearly twice as great as that of *M. hubbsi* and equal to that of the European species. It seems quite probable, from the known distribution of ocean temperatures during the colder part of the year, that rumours of the presence of *M. gayi* on the equator, at the Galapagos Islands, may prove well founded. I cannot confirm this from the literature available, but the dubious specimens of *Merluccius* recorded from Panama Bay (7° N.) by Garman (1899) as *M. angustimanus* probably represent a race or 'phase' of *M. gayi*. This is the view of Norman (1937), and Hildebrand (1946) seems to agree with him. Off the Chilean and Peruvian coasts the Peru coastal current produces negative temperature anomaly throughout the equatorial half of the range of *M. gayi*, on the greatest scale known. In the polar half of its range the species is known to be abundant down to Talcahuano (37° S.), where it is killed in large numbers by the putrefaction resulting from the periodic mass suicide of large squids (Gunther, 1936), and smaller numbers are known to occur for at least a further 10° to the southward. Here the hydrological features do not appear specially favourable to its extended distribution, but the absence of shoal-water along this coast may render the subsurface currents (of which we know but little) of greater relative importance here.

It should of course be realized that subsurface currents almost certainly influence hake distribution elsewhere, especially off New England (*M. bilinearis*), but we do not yet know enough about them to assess their effect with any certainty. It is the whole complex of environmental factors, more or less intimately related to the primary influences of temperature and current in the surface layers (where the phytoplankton production determines the ultimate food supply of any given sea area), that we are really dealing with in this survey of hake distribution. The surface temperatures merely supply the most adequate body of data broadly symptomatic of the complex.

The distribution of the Californian hake, *M. productus*, is less well known, for there is no regular fishery for it on those favoured coasts where choicer food fishes abound. Clemans & Wilby (1946) give its range as southern California to northwestern Alaska. It is probable that stragglers may extend across the North Pacific to the Asiatic side, but the species is not mentioned in recent extensive Russian work. Farther south there is a doubtful record from the China coast by Bleeker (1873) which Chu (1931) is inclined to disregard. I have not been able to consult all the references relating to this point which are given by Fowler (1933). There seems little reason to doubt that a fuller knowledge of the distribution of *M. productus* will serve only to emphasize a relation to major hydrological features similar to that which we have been able to trace for better-known species of hake elsewhere.

The distribution of New Zealand hake is also little known. It seems to extend all round South Island and is found at the Chatham Islands. Phillipps (1921) says of the species: 'Odd examples as far north as Hawke Bay...unknown north of East Cape.' *M. australis* was not found during earlier work at any of the sub-antarctic islands of New Zealand, but as Waite (1909) remarks, the deeper waters were not then netted. Trawling will probably reveal its presence at some of them eventually.

It is somewhat surprising that *M. australis* seems not to extend all round North Island, for from the known distribution of the other species elsewhere there would seem to be no hydrological barrier. Certainly the temperatures seem sufficiently moderate, but in a region where an ample supply of choicer fish can be obtained by small-scale methods, mostly operated well inshore, it is obvious that we have insufficient evidence as yet fully to consider the distribution of such fish as hake.

M. australis nevertheless introduces us to two of the most enigmatic features of the distribution of the genus as a whole. First, Norman (1937) could find no differences between recent New Zealand specimens and one recorded as taken by the *Challenger* in Messier Channel (western Magellan region!). This seems to be the only well-authenticated instance of a hake species straggling across any of the great oceans in west to east direction in the southern hemisphere, and the distance involved is the greatest attainable. Secondly, hake are unknown in Australian waters, though one would have thought the hydrological conditions quite suitable for them, especially on the banks south-east of Tasmania and near Cape Leeuwin.

There is just a possibility that hake may be found round the islands of St Paul in the Indian Ocean—possibly a race of *M. capensis*. We have no evidence of this beyond the apparent hydrological suitability of the surrounding seas, but here is one of the many reasons why the study of the fish faunas of these and other isolated oceanic island groups may yield results of outstanding interest.

VII. SUMMARY

A brief account of some of the recent advances in our knowledge of the general biology, taxonomy and economic importance of the genus *Merluccius*, which have resulted mainly from the work of Hickling and of the *Discovery* investigations, has been given by way of introduction to a more detailed discussion of its distribution, and in the hope of demonstrating the great interest of hake from a general biological point of view.

The seven species of true hakes (i.e. not *Urophycis* spp.) distinguished by Norman (1937), and their normal distributional limits, are:

Merluccius merluccius (Linnaeus)	From the Norwegian Rinne southwards along the edge of the continental shelf west of Europe to Dakar. Mediterranean.
M. hubbsi Marini	From Magellan Straits northwards over the Patagonian continental shelf to southern Brazil.
M. productus (Ayres)	Southern California to north-western Alaska. ? China.
M. gayi (Guichenot)	Southern Chile to Paita in Peru, and possibly farther north.
M. bilinearis (Mitchill)	South coast of Newfoundland southwards to North Carolina and, rarely (in deep water only) to Florida and the Bahamas.

M. capensis Castelnau	Off South Africa, from Angola to Natal.
M. australis (Hutton)	Chatham Island, South Island of New Zealand and northwards to East Cape on North Island. A *Challenger* specimen from the Magellan region.

There is a remote possibility that certain aberrant specimens secured in deep tropical waters off West Africa and in Panama Bay may represent two further distinct species, bridging the gaps between *M. merluccius* and *M. capensis* and between *M. gayi* and *M. productus*. It is more probable that they represent odd stragglers (at most only racially distinct) of *M. merluccius* and *M. gayi* respectively.

It has been shown that all the best-known species of *Merluccius* conform in a striking manner to the same distributional pattern in relation to the major hydrological features, within the limits of their normal range. Where relatively cold currents flow towards the equator in the warmer half of the normal habitat of any one of these species, the range of the species is extended in that direction; but if a relatively warm current is flowing polewards, the range in the direction of the equator is restricted. In the colder half of the normal habitat of each species the converse relationship holds good.

Surface isotherms have been used as the most reliable general criterion symptomatic of the environmental complex that leads to this type of distribution because in many parts of the world more detailed hydrological data are not yet available; but it is emphasized that other factors, more or less intimately interrelated with the direct effect of temperature, are also involved.

The distribution of the genus *Merluccius* seems to offer a good example of the wider aspects of the phenomenon of 'organic polarity' discussed by Wimpenny (1941), while the bionomics of the better known individual species show some more detailed aspects of it with great clarity.

I am greatly indebted to Dr N. A. Mackintosh, Director of Research, *Discovery* Investigations, for permission to publish this review while I am still engaged on other work for the *Discovery* Committee, and to Miss E. Humphrey, who re-drew the chart for publication; also to the Director and staff of the Marine Biological Association's Plymouth Laboratory (where the article was written), especially the Librarian, Miss Sexton. Talks with Messrs Hickling, Carruthers, Deacon and Wimpenny have been most helpful and stimulating.

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ECHINODERM EMBRYOLOGY AND THE ORIGIN OF CHORDATES

By H. BARRACLOUGH FELL
Victoria University College, Wellington, New Zealand

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In this article it is proposed to review some recent researches in echinoderm embryology, with special reference to their bearing on the evolution, specialization and omission of larval forms, together with an appraisal of the embryological evidence for the supposed echinoderm origin of chordates.

I. THE PLASTICITY OF THE LARVAL STAGE

1. *Larval morphology*

The fundamental features of the development and interrelationships of echinoderm larvae, as worked out during the latter part of the nineteenth century, were derived from the initial brilliant researches of Johannes Mueller (1846 *et seq.*) who demonstrated that the four main types arise in each case from a simple *dipleurula* form. Ignoring at present the coelom, which is discussed later, the basic plan of development is represented in Fig. 1. The dipleurula arises from a preceding gastrula stage by the formation of a ciliated (or vibratile) band (Fig. 1 A) which forms a closed loop about the mouth. A simple alimentary canal is present, comprising stomodaeum, archenteron or stomach, and proctodaeum; the anus is generally, but not always, formed from the blastopore. In its subsequent development the dipleurula undergoes a varying transformation according to the systematic position of the parent species.

In the classes Echinoidea and Ophiuroidea the development of paired arms or processes on either side of the body, upon which the ciliated band becomes extended, leads to the formation of the *simple pluteus* (Fig. 1 B). Further development of paired arms, strengthened by internal calcareous rods, leads to the final larval forms, distinguished by Mortensen (1898) as *echinopluteus* and *ophiopluteus* according to the class.

The echinopluteus usually possesses four pairs of arms (Fig. 1 C), though more or fewer may be present in certain forms. The four principal pairs of arms comprise

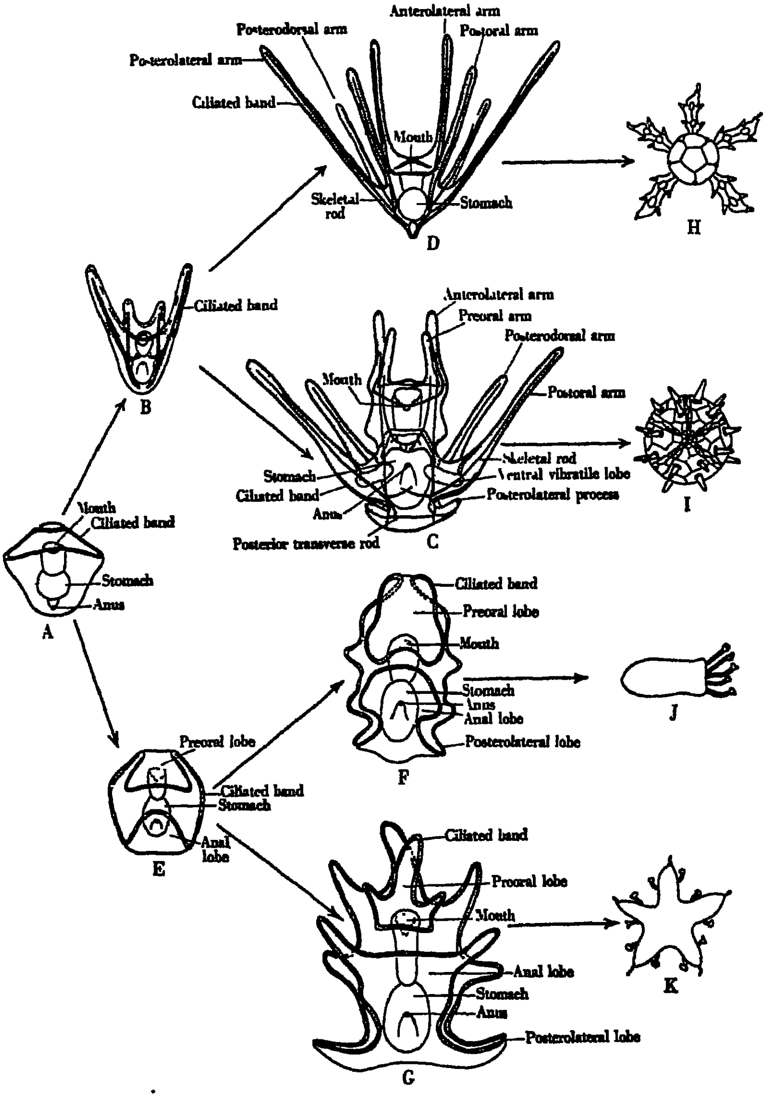

Fig. 1. General scheme indicating the relationships of bilaterally symmetrical echinoderm larvae. A, dipleurula. B, pluteus. C, echinopluteus, which metamorphoses into a young echinoid, I. D, ophiopluteus, which metamorphoses into a young ophiuroid, H. E, early auricularia. F, fully developed auricularia, which metamorphoses into a young holothurian, J. G, bipinnaria, which metamorphoses into a young asteroid, K.

two anterior pairs, the anterolateral and preoral pairs, and two posterior pairs, the postoral and posterodorsal pairs. In addition, at the extreme posterior end of the body are commonly found a pair of posterolateral processes, which are occasionally elongated to form distinct posterolateral arms. Anterodorsal arms occur in certain cases as a still further complexity (compare Fig. 5 I, a spatangoid echinopluteus). These various arms are supported by slender calcareous rods, which may form a mesh about the archenteron at their inner extremities within the body; often a special posterior transverse rod is formed between the posterolateral processes. Certain portions of the ciliated band may become thickened dorsally and ventrally to form the paired dorsal and ventral vibratile lobes; or these may become separated from the main band to form prominent epaulets, as in Fig. 5 G.

The ophiopluteus (Fig. 1 D), if fully developed, is of rather similar appearance, having four pairs of arms, which however do not correspond altogether with the four pairs commonly present in the echinopluteus. It is usually the case that the most prominent and enduring arms are those which are termed the posterolateral pair, which Mortensen has shown to be homologous with the posterolateral lobes of the echinopluteus as normally formed. The other arms are the anterolateral, the postoral and posterodorsal pairs respectively. The preoral arms of the echinopluteus are not represented. The internal skeleton takes the form of a pair of calcareous rods in the body, each sending branches into the four arms on its corresponding side; the posterior transverse rod is not represented.

In the classes Holothuroidea and Asteroidea no comparable development of paired arms occurs. Initially the dipleurula becomes rather barrel-shaped (Fig. 1 E), and the ciliated band is thrown into folds in an anterior and posterior loop. Further sinuous growth of the ciliated band leads to the characteristic larval forms, the *auricularia* in holothurians, and the *bipinnaria* in asteroids.

The auricularia (Fig. 1 F) is characterized by the formation of two lobes bordered by the folded ciliated band, the preoral lobe anteriorly, and the anal lobe posteriorly, the latter bearing the anus. The mouth lies in a depression on the ventral side, between the preoral and anal lobes. Smaller posterolateral lobes occur in the same relative position as in the preceding larvae.

The bipinnaria is much more variable in structure. Sometimes, especially in primitive starfish, it is scarcely distinguishable from the simple auricularia. More frequently the sinuous lobes of the ciliated band become drawn out into prominent arms (Fig. 1 G) which, however, lack skeletal rods. Consequently they are not rigid, and are utilized as flexible swimming organs. Posterolateral lobes are usually found as in the auricularia. Median arms often appear anteriorly to the mouth, on the preoral lobe. It is usual for the bipinnaria stage to be followed by what is termed a *brachiolaria* larva, distinguished principally by the development of median sucking arms (or disk), anterior to the mouth, by means of which the larva may become temporarily attached.

In each case metamorphosis involves either discarding or absorbing the paired larval structures, while a secondary radial symmetry is initiated through encirclement of the gut by the five lobes of the hydrocoel.

The embryology of crinoids, so far as is known, does not include a comparable bilaterally symmetrical larva, and the mode of development is so different that it cannot be treated in the scheme above. As will be further noted later on, a large proportion of echinoderms other than crinoids also undergo developments of an entirely different character from the basic plan outlined above. A frequent condition in forms with large yolky eggs is that the larva is a simple cylindrical form with a number of transverse ciliated bands. This type, which is sluggish and does not take food, has been termed the *vitellaria*. It is formed variously, and the internal organogeny differs according to the class.

Echinoderm larvae occasionally grow to a considerable size. Thus the bipinnaria of *Luidia sarsi*, i.e. the *Bipinnaria asterigera* of Sars (1835), reaches a length of 2·4 cm. A New Zealand form, *Auricularia nudibranchiata* of unknown parentage, measures at least 1 cm. in length (MacBride, 1920).

The above facts offer interesting problems. The general occurrence of a pelagic bilaterally symmetrical dipleurula stage in four of the existing classes has been interpreted as a recapitulation of a hypothetical dipleurula form, from which all echinoderms are supposed to have descended. This view has not been seriously questioned and remains acceptable to taxonomists and embryologists alike. But beyond this point matters are less clear. Are the succeeding larval stages to be interpreted in the same light? If so, we would be led to the conclusion that ophiuroids and echinoids, with their similar pluteus stages, are more closely related to each other than to any of the remaining classes. Neither taxonomic nor palaeontological evidence supports such a deduction. There can be little doubt that the ophiuroids are related to the asteroids through more generalized Palaeozoic stelleroids. The alternative is to regard the characteristic post-dipleurula larval stages as 'digressions' from the original path of development, which have arisen independently in the various classes by clandestine evolution—to use de Beer's term (1930) for evolution which does not affect adult forms. Mortensen (1921) regards these larvae as highly specialized adaptations of the original pelagic ancestral form, having been modified along with the adults, so as to form groups corresponding with the natural groups of the adults. I have suggested (Fell, 1945) that they have arisen in response to the need for a temporary food-gathering stage as a preliminary to further development; for they almost invariably arise from eggs which are deficient in yolk and cytoplasm. A more specific examination of modes of echinoderm development also leads inevitably to the conclusion that special larval evolution has occurred, often quite independently of natural groupings, as the following cases illustrate.

2. *Larval convergence*

It is instructive to compare the larval development of an ophiuroid such as *Ophiothrix*, well known through the work of MacBride (1907), with that of echinoids of the genus *Centrechinus* (*Diadema*) described by Mortensen (1931). The echinopluteus of the latter is greatly modified through unusual development of the postoral arms and reduction of the other arms, so that it superficially resembles an ophiopluteus (Fig. 2). This case, where adults so conspicuously distinct, and only remotely related, possess such surprisingly similar larval forms, permits of only one interpretation: convergent embryonic evolution has occurred in the echinoid, which temporarily resembles an ophiuroid stage.

Even more striking is the case of the vitellaria larva (Fig. 3). This characteristic cylindrical larva with ciliated annulations, derived from a yolky egg, occurs in three classes, the holothurians and crinoids commonly, and the ophiuroids rarely. Although Grave in 1903 regarded this larva as representing an original primitive form, such a view cannot be regarded as probable, for many obvious reasons.

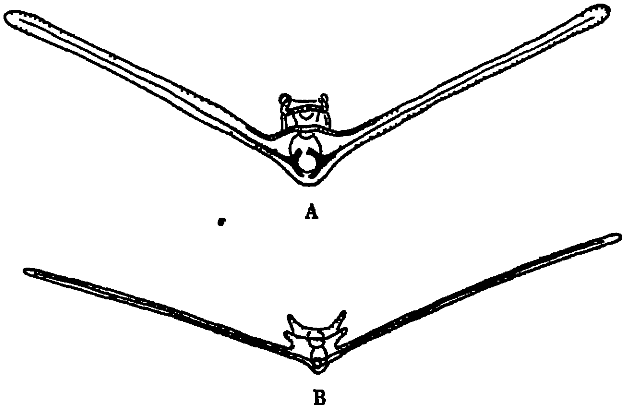

Fig. 2. Convergent larval forms. A, an echinoid, *Centrechinus* (*Diadema*). B, an ophiuroid, *Ophiothrix*.

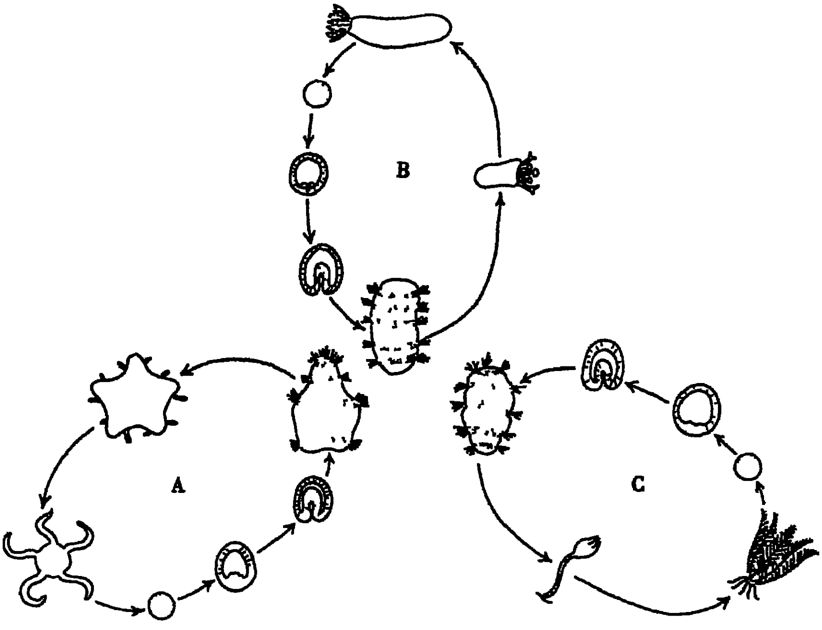

Fig. 3. Convergent development of unrelated echinoderms. A, class Ophiuroidea (*Ophioderma*). B, class Holothuroidea (*Cucumaria*). C, class Crinoidea (*Antedon*). In each case the larva is a vitellaria.

3. *Larval divergence*

Divergent evolution in larval development is strikingly illustrated throughout the phylum. In ophiuroids, for example, quite diverse modes of development occur (Fig. 4). The case of *Amphiura* is illustrative. Two closely related species in this genus develop in so diverse a manner, that, were the adults unknown, the young stages could hardly be recognized as belonging to the same class, certainly not to the same genus. Nor is this an isolated case. In *Ophioderma brevispina*, studied by Grave in 1900, the larva is a vitellaria. In the not distantly related *Ophiura texturata* there is a well-developed ophiopluteus, with four pairs of arms. In other species such as *Ophiomyxa brevirima* there is probably no larva at all, and certainly there is no vestige of a bilateral larva in Kirk's ophiuroid (Fell, 1941 *a*, *b*). These variations are not exceptional, for it can no longer be maintained that any particular mode of development is general among ophiuroids, a point discussed further below.

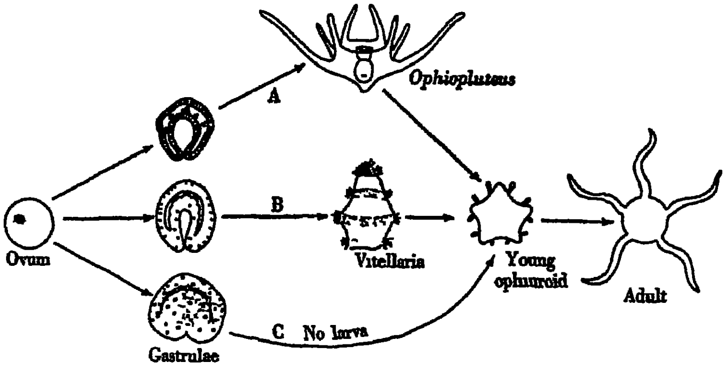

Fig. 4. Divergent modes of development in ophiuroids. A, *Ophiothrix*. B, *Ophioderma*. C, Kirk's ophiuroid.

4. *Environmental responses*

The remarkable modifications of structure which can be evoked in echinoderm larvae by relatively insignificant changes in the salinity, hydrogen ion concentration, oxygen content, etc., of the environment are well known. It is not within the scope of this review to discuss experimental embryology. However, in nature equally striking responses can be observed. Thus, in the viviparous ophiuroid *Amphipholis* embryonic attachment organs occur in those stages which are passed within the bursa of the parent. The profound changes attributable to the effect of the internal environment of the embryo are considered later.

5. *Conclusions*

Embryos and larvae of echinoderms are essentially plastic and susceptible to evolutionary modifications of structure which may act independently of the adult stage. For this reason phylogenetic deductions should not be based on larval forms alone.

Before considering possible causes for such modifications it is necessary to review some remarkable recent researches which have demonstrated how embryological evolution has occurred independently yet in parallel with adult evolution.

II. ECHINODERM LARVAE AND TAXONOMY

Researches leading to the conclusion that definite relationships can exist between the structure of adult echinoderms and that of their larvae have been carried out by Mortensen (1921 and later references). Thus in the Echinoidea the extant orders, and frequently families, can be characterized by the nature of the echinopluteus. To a lesser extent a similar relation is seen in Asteroidea. The position in the other classes is not so clear. These results, in conjunction with other relevant studies, are summarized in the following survey.

1. *Class ECHINOIDEA*

Order CIDAROIDEA

Until 1937 the development of cidaroids was known only very incompletely, and no general conclusions could be drawn as to its nature. The following species had been studied: *Cidaris cidaris* (Prouho, 1888), *Eucidaris tribuloides* (Tennant, 1922) and *E. thouarsii* (Mortensen, 1921). In addition, Mortensen (1921) had shown that the development of *Phyllacanthus parvispinus* is probably direct. In 1937 Mortensen published his account of *Eucidaris metularia*, and the following year that of *Prionocidaris baculosa*, which was studied through metamorphosis. The two latter accounts are the most important, and together with the earlier data enabled the conclusion to be drawn that there is a distinctive cidaroid larval form (Fig. 5B). This is an echinopluteus characterized by the very long, smooth fenestrated postoral and posterodorsal rods; by having the posterior transverse rod provided with long, slender median processes dorsally and ventrally; and by the strong development of the ciliated lobes. A larval muscular system is present in the two latter species, enabling the two pairs of long arms to be moved backwards and forwards, and no doubt this feature is also generally characteristic of the cidaroid larva.

A further feature of morphological interest is that in the newly metamorphosed urchin of *Prionocidaris* a pluriserial interambulacrum occurs, as in Palaeozoic echinoids. These are later resorbed, as in *Archaeocidaris*, thus lending support to Mortensen's contention that *Archaeocidaris* and not *Bothriocidaris* is to be regarded as ancestral to the Cidaroidea.

Order CENTRECHINOIDEA (DIADEMOIDEA)

In this group of echinoids distinctive echinoplutei can be correlated with the families Centrechinidae (Diadematidae) and Arbaciidae. The development of the Echinothuridae and Salenidae remains unknown.

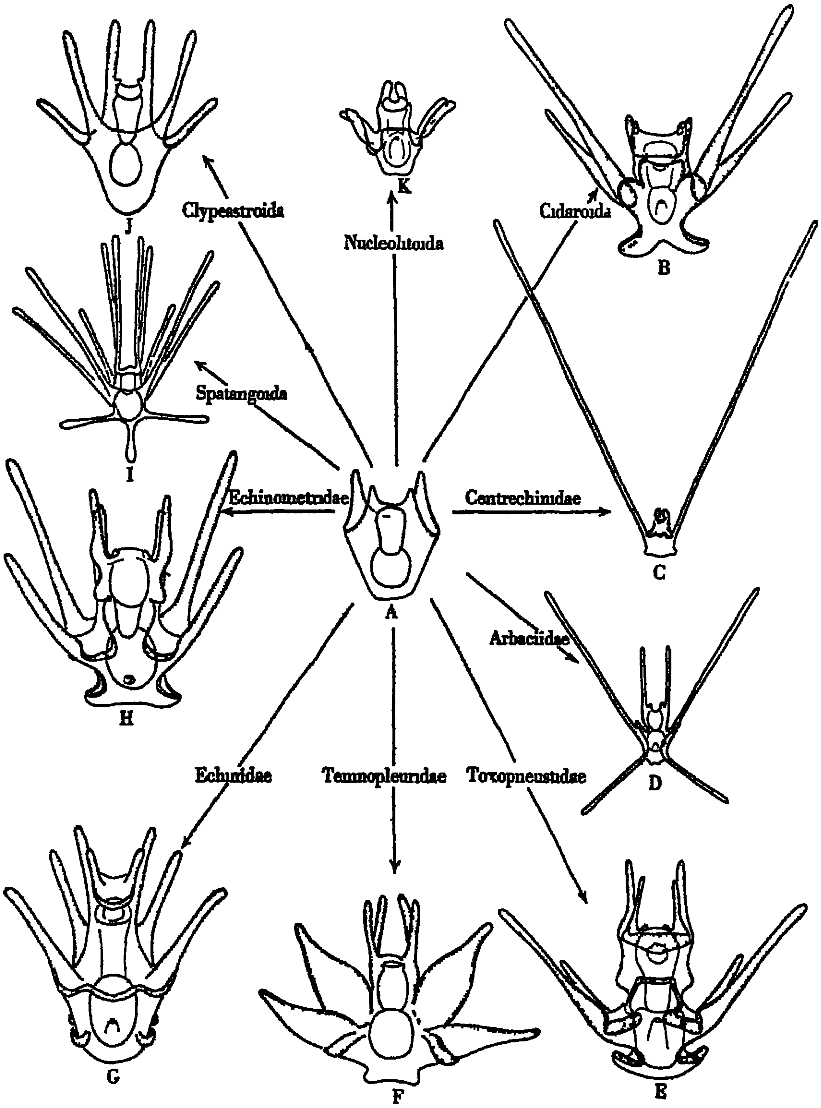

Fig. 5. Divergent echinoid larvae showing larval evolution corresponding to natural taxonomic groups based on adult forms. All are echinoplutei derived from the basal early pluteus form. For further details see text.

Family CENTRECHINIDAE

Knowledge of the development of these is due mainly to Mortensen. The following species have been investigated: *Centrechinus* (*Diadema*) *setosum* (Mortensen, 1931, and Onoda, 1936), *C. antillarum*, *C. savignyi* and *Echinothrix diadema* (Mortensen, 1931). It is now evident that the larva of these (Fig. 5 C) is of the form earlier characterized under the name *Echinopluteus transversus* (Mortensen, 1921) the parental species being at that time unknown. It is notable for the great development of the postoral arms, the other arms being absent save for small anterolaterals which may be present. The larva thus strongly resembles an ophiopluteus. The ciliated band is not strongly developed, in sympathy with the reduction of the arms, and there are no vibratile lobes or epaulets. There are also other skeletal peculiarities which need not be specified here.

Family ARBACIIDAE

The development of three species of the genus *Arbacia* has been studied: *A. lixula* (J. Mueller, 1853; *et al.*); *A. punctulata* (Fewkes, 1880; Brooks, 1882; Garman & Colton, 1882); *A. stellata* (Mortensen, 1921). The arbaciid larva (Fig. 5D) has at first a basket skeleton. In its second stage of development a posterior transverse rod is present ending in a pair of long posterolateral arms which are similar to the other arms. Postoral and posterodorsal rods are fenestrated. There are large ventral and dorsal lobes, but no epaulets.

Order CAMARODONTA

Distinctive echinoplutei are known for each of the families Temnopleuridae, Echinidae, Toxopneustidae and Echinometridae (using these in the sense proposed by Mortensen (1921), who regards *Toxopneustes* and its allies as constituting a distinct family, restricting the other families accordingly).

Family TOXOPNEUSTIDAE

Knowledge of the development of these is derived from studies of the following species: *Lytechinus variegatus* (Tennant, 1910; Mortensen, 1921); *L. anamesus*, *L. pictus*, *L. panamensis* (Mortensen, 1921); *L. verruculatus* (Mortensen, 1921, 1931); *Nudechinus gravieri* (Mortensen, 1937); *Toxopneustes pileolus* (Mortensen, 1921; Onoda, 1936); *T. roseus* (Mortensen, 1921); *Tripneustes esculentus* (Tennant, 1910; Mortensen, 1921); *T. gratilla* (Mortensen, 1921, 1931, 1937; Onoda, 1936); *Sphaerechinus granularis* (J. Mueller, 1855, *et al.*); *Pseudocentrotus depressus* (Mortensen, 1921; Onoda, 1936). Further, if *Strongylocentrotus* be admitted to this family, as Mortensen has suggested, the following four species may be added: *S. droebachiensis* (A. Agassiz, 1864); *S. franciscanus* (Loeb, 1908; Hagedoorn, 1909; Mortensen, 1921; Johnson, 1930); *S. purpuratus* (Hagedoorn, 1909); *S. pulcherrimus* (Mortensen, 1921; Onoda, 1936).

The echinopluteus of this assemblage possesses in its first stage a short body, usually with a basket structure of the skeleton. In its second stage (Fig. 5E) there is a posterior transverse rod. Posterolateral arms occur, and also vibratile lobes, and epaulets at the bases of the four main arms. The rods of the main arms may be either simple or fenestrated (Mortensen, 1921).

Family TEMNOPLEURIDAE

The following species have been studied: *Temnopleurus toreumaticus* (Mortensen, 1921; Onoda, 1936); *T. hardwickii* (Moore, 1933); *Temnotrema sculpta* (Mortensen, 1921); *T. scillae* (Mortensen, 1937); *Mespilia globulus* (Mortensen, 1921; Onoda, 1936); *Salmacis bicolor* (Aiyar, 1935).

In the first stage there is no basket structure of the skeleton. In the second stage (Fig. 5 F) a posterior transverse rod forms, from which short branched posterolateral rods may or may not issue. There are four vibratile epaulets, but no vibratile lobes. The arms are often highly characteristic owing to a bloated condition (Mortensen, 1921).

Family ECHINIDAE

The development of several species of this family has been well known for a long time, although it was not till 1921 that Mortensen first defined the type of echinopluteus characteristic of the family. The following species have been studied: *Paracentrotus lividus* (J. Mueller, 1852; Metschnikoff, 1869 *et al.*); *Psammechinus miliaris* (MacBride, 1898; Mortensen, 1898; Theel, 1902 *et al.*); *P. microtuberculatus* (J. Mueller, 1852; Selenka, 1879; Giesbrecht, 1913); *Echinus esculentus* (MacBride, 1898, 1903; Shearer, de Morgan & Fuchs, 1914); *Parechinus angulosus* (Mortensen, 1931); *Echinus acutus* (Shearer, de Morgan & Fuchs, 1914); *Sterechinus agassizi* (Mortensen, 1913).

In its first stage the larva has a short body, usually with a basket structure of the skeleton. In the second stage (Fig. 5 G) there is a posterior transverse rod; posterolateral and vibratile lobes occur, and also epaulets at the bases of the four main arms; the rods of the main arms may be either simple or fenestrated.

Family ECHINOMETRIDAE

The larval development of this family remained unknown until 1921, when Mortensen published accounts of nine species. Onoda (1931, 1936) has since added information on two other species. The following have been studied: *Echinometra lucunter* (Mortensen, 1921); *E. oblonga* (Mortensen, 1921); *Colobocentrotus astratus* (Mortensen, 1921); *Echinometra mathaei* (Mortensen, 1921, 1937; Onoda, 1936); *Heterocentrotus mammillatus* (Mortensen, 1921, 1937); *Heliocidaris tuberculata* (Mortensen, 1921); *H. crassispina* (Onoda, 1931); *Evechinus chloroticus* (Mortensen, 1921); *Stomopneustes variolaris* (Mortensen, 1931); *Echinostrephus molaris* (Onoda, 1936). To these may be added *Heliocidaris erythrogramma* for which Mortensen (1921) has given a partial account indicating that a form of direct development occurs.

The echinometrid larva has in the first stage the body short and obliquely truncate, supported by a complex basket structure. In its second stage (Fig. 5 H) there is a posterior transverse rod present. Posterolateral and vibratile lobes occur, but no epaulets. The rods of the main arms are fenestrated (Mortensen, 1921).

Order NUCLEOLITOIDA

There are only two surviving species of this Mesozoic and early Tertiary group; of these *Apatopygus* (*Echinobrissus*) *recens* is found in New Zealand and Mortensen (1921) has given an account of the larval development, which appears to indicate affinity with the clypeastroids. The fully developed larva is as yet unknown, but Mortensen's oldest stage is illustrated in Fig. 5K.

Order CLYPEASTROIDEA

A series of fifteen species is available for comparison of their larval development, which has been shown to be of a characteristic type (Mortensen, 1921). The species are: *Echinocyamus pusillus* (Theel, 1892; Mortensen, 1931); *Fibularia craniolaris* (Mortensen, 1937); *Echinarachnius parma* (A. Agassiz, 1864; Fewkes, 1886); *E. excentricus* (Mortensen, 1921); *Peronella leseuri* (Mortensen, 1914; Tennant, 1915; Mortensen, 1921); *Clypeaster japonicus* (Mortensen, 1921); *C. humilis* (Mortensen, 1937); *Arachnoides zelandiae* (Mortensen, 1921); *A. placenta* (Feliciano, 1933); *Encope micropora* (Mortensen, 1921); *Mellita sexiesperforata* (Mortensen, 1921); *Astriclypeus manni* (Mortensen, 1921); *Laganum diplopora* (Mortensen, 1921); *L. depressum* (Mortensen, 1938); *Echinodiscus auritus* (Mortensen, 1937, 1938).

In the clypeastroid larva the body skeleton forms a prominent basket structure of distinctive character. This often takes the form of a large, complex, fenestrated plate in the posterior end of the body (Fig. 5J).

Order SPATANGOIDEA

As is well known, the spatangoids are characterized by possessing a highly distinctive echinopluteus which bears a median unpaired arm-like process (Fig. 5I). In addition, the anterodorsal arms are well developed. Mortensen (1921) has further suggested that it may ultimately be found that the two families Spatangidae and Brissidae have distinctive larvae, as from the data available it appears that the former possess posterolateral arms, while the latter lack these structures.

The following spatangoid larvae are known: Spatangidae—*Spatangus purpureus* (Krohn, 1853; Mortensen, 1913); *Echinocardium cordatum* (Mortensen, 1898, 1931; MacBride, 1913); *E. australe* (Mortensen, 1921); *Moira atropos* (Grave, 1902; Tennant, 1910); *Lovenia elongata* (Mortensen, 1937). Brissidae—*Brissus obesus*, *B. agassizi*, *Brissopsis lyrifera* (Mortensen, 1921); and, doubtfully identified, *Meoma grandis* (Mortensen, 1921).

2. *Phylogenetic implications of echinoid development*

The conclusions drawn from the above data by Mortensen may be quoted as given substantially in his main study (1921):

> The Echinopluteus affords the greatest diversity of forms of all Echinoderm larvae, and several well-marked types are to be distinguished. It does not immediately appear which of these represents the more primitive type. Considering, however, the fact that the larvae in which the body skeleton in the first stage forms a basket structure, and which have in their second stage a posterior transverse rod and more or less developed posterolateral processes are characteristic—so far as we know—of the Cidarids, Diadematids and Arbaciids, that is to say of the more primitive forms of Echinoids, it can hardly be disputed that we

> have got to regard this larval type as the more primitive form. *Consequently the larval type characteristic of the family Echinidae s. str.*, with the elongated, club-shaped body rods, with the recurrent rod rudimentary or absent, and without a posterior transverse rod or posterolateral processes, *is highly specialized.* It is therefore not at all justifiable to make this larval type represent the Echinoid larva in general as is done in most text-books.
>
> Characteristic of the larval body of the primary type are the vibratile lobes; in the more specialized types, the larva of the Echinidae *s. str.*, and of the Spatangoids, these lobes have disappeared, while the Clypeastroid larvae have retained them to some degree. A further specialization from the lobes are the epaulets occurring in the higher types of the Regularia.
>
> Both the vibratile lobes and the epaulets evidently serve to increase the floating power of the larva. This object is attained to a still higher degree in several larval forms of Regular Echini and Clypeastroids in which muscles connect the lower ends of the rods of the four main arms, so that these arms become actively movable. These larvae, when floating, keep the four main arms in a more or less horizontal position, raising them when disturbed. This is not yet an active swimming movement, the muscular apparatus being too simple for performing regularly repeated movements. Only one Echinoid larva appears to be able to swim actively, viz. the remarkable *Echinopluteus transversus* (of Centrechinidae—H. B. F.), in which a complicate muscular system has developed, the body-skeleton being most extraordinarily adapted for serving as a support to the muscles....
>
> As regards the skeleton it is a noteworthy fact that the fenestrated rods represent a primary structure, as must be concluded from the fact that this type of rods (always confined to the four main arms) is found in the larvae of the more primitive forms....

So far as concerns the broader aspect of this review, a logical conclusion deducible from the array of facts presented is that larval evolution of the echinopluteus must have occurred subsequently to the separation of the main orders and families of echinoids, and within any one group of echinoids the larval evolution has followed similar trends. Thus, within relatively small groupings it is true to say that the young stages of related species show similar ontogenies. It is obvious that, with independent larval evolution occurring simultaneously in the many orders and families of echinoderms in general, the phylogenetic relationship between major groups, such as the classes, will become ever more and more obscured in so far as it is reflected in embryology. The point becomes most important when it is proposed to base relationships between the echinoderm and chordate phyla on evidence obtained from a few selected larval forms which cannot be proved to have any phylogenetic significance. Further discussion of the point must be left to the final part of this article.

3. *Class ASTEROIDEA*

The larva if present in development is, initially at least, always a bipinnaria. A more complex larval form, the brachiolaria, usually follows. The two families Astropectinidae and Luidiidae stand apart from all others in that the larva never, so far as is known, proceeds beyond the bipinnaria stage. All other asteroids appear to possess a brachiolaria, though this may be much reduced in cases of direct development (Mortensen, 1938). Variations in larval development of asteroids, so far as they can be correlated taxonomically, may be summarized in the following way.

Order PHANEROZONIA

Family ASTROPECTINIDAE

The larval forms of five species of *Astropecten* are known with certainty, and all are so similar as to be scarcely distinguishable. Mortensen concludes that the Astropectinidae possess a distinctive larva, which is a bipinnaria having the special characteristics of broad, round anterior lobes. There is no brachiolaria. The larval body is completely absorbed during metamorphosis.

The species studied are: *Astropecten aranciacus* (Metschnikoff, 1885; Hörstadius, 1926); *A. pentacanthus* (Metschnikoff, 1885); *A. scoparius* (Mortensen, 1921); *A. polyacanthus* (Mortensen, 1921, 1937); *A. velitaris* (Mortensen, 1937).

Family LUIDIIDAE

The general characters of the *Luidia* larva appear to be: a more or less pronounced elongation of the anterior part, the median lobes; the total absence of brachiolarian arms and sucking disk. It differs from the *Astropecten* larva by the more developed arms, and the elongation of the median lobes.

Species studied are: *Luidia sarsi* (Meek, 1927; Tattersall & Sheppart, 1934); *L. ciliaris* (Mortensen, 1898, 1913; Gemmill, 1916); *L. savignyi* (Mortensen, 1938).

Other Asteroidea

The remaining families of the Phanerozonia, as well as the other two orders Spinulosa and Forcipulata, possess a brachiolaria or vestige of such a stage.

The following Phanerozonia have been studied: *Pentaceraster mammillatus* (Mortensen, 1938); *Linckia multifora* (Mortensen, 1938); *Asterope carinifera*; *Porania pulvillus* (Gemmill, 1915); *Gymnasteria carinifera* (Mortensen, 1921); *Archaster typicus* (Mortensen, 1921); *Acanthaster planci* (Mortensen, 1931); *Culcita schmiedleriana* (Mortensen, 1931); *Cheiraster gerlachii* (MacBride, 1920); a brachiolaria stage has been established for all of these save the latter species, which is doubtful.

Among the Spinulosa partially direct development, without a pelagic stage, is common. Pelagic brachiolariae are known from the species *Asterina pectinifera* (Mortensen, 1921); *Patiria miniata* (Heath, 1917; Newman, 1925); *Stichaster roseus* (Gemmill, 1916; Mortensen, 1921). In *Asterina regularis* the development has been followed only to the bipinnaria stage (Mortensen, 1921), but no evidence was found to suggest that a brachiolaria stage is omitted. *Brisaster fragilis*, studied by Runnström, is a notable exception to the general rule in possessing large yolky eggs which nevertheless develop into a pelagic larval form, which is a brachiolaria (see below on the relation of egg size to mode of development).

Spinulosa possessing large yolky eggs and having a more or less direct development include species of *Asterina*, *Solaster*, *Fromia* and *Echinaster*, to which reference is made later.

Forcipulata studied include: *Asterias rubens* (Mortensen, 1898; Gemmill, 1916); *A. forbesi* (A. Agassiz, 1877); *A. vulgaris* (A. Agassiz, 1877; Goto, 1896): *Coscinasterias calamaria* (Mortensen, 1921); *Marthasterias glacialis* (Russo, 1892; Mortensen, 1913; Gemmill, 1916). The brachiolaria is the normal larva.

4. *Phylogenetic implications of asteroid development*

The significance of the above facts has been variously interpreted.

MacBride, who regarded the brachiolaria as the more primitive of the two larval forms, considered the sucking disk of the brachiolaria to be homologous with the stalk of an attached pelmatozoon. He regarded the temporary period of attachment in the life of asteroids, followed by the free radial form, as an ontogenetic recapitulation of the evolutionary history of the class.

In opposition to the above view Mortensen (1921) pointed out that the brachiolaria larva is restricted to the more specialized Asteroidea (i.e. the Spinulosa and Forcipulata) while the more primitive Phanerozonia lack the larva (the fact that Phanerozonia such as *Archaster* possess a brachiolaria being unknown at that date). He also considered that the brachiolarian arms and sucking disk are essentially specialized structures, the more so since they arise relatively late in development, subsequent to the bipinnaria stage. He considered the bipinnaria to be the primitive form, and that phylogenetic speculations based on brachiolarian structures cannot be held valid.

MacBride (1921) in reply stated his belief that the Spinulosa, not the Phanerozonia, are the more primitive asteroids, and he explained the absence of an attached stage in the Phanerozonia as consequent upon sandy or muddy habitats not permitting such a stage. In the former contention MacBride is not supported by the majority of taxonomists of the group.

W. K. Fisher, H. L. Clark, R. Koehler and L. Doderlein all agreed that the Astropectinidae are most primitive. MacBride and Perrier were alone in regarding the Spinulosa as occupying this place. Gemmill (1923) stated that he did not regard the Astropectinidae as primitive, and considered that the Asterinidae should be placed in the Phanerozonia. The latter opinion, of course, is again contrary to the views of most taxonomists.

Bather (1923) gave it as his view that, assuming Mortensen to be correct in asserting that only the more specialized forms possess the sucking disk, the sucker may none the less perpetuate ancestral structure.

MacBride in 1923 repeated his opinion that the crinoid larva and brachiolaria are comparable: in both there occur the long preoral lobe, ventral stomodaeum, right and left posterior coeloms. In both the preoral lobe becomes the stalk. He regarded the stalk as homologous in each case.

Hörstadius (1926), following his study of the development of *Astropecten*, considered that the bipinnaria is more primitive than the brachiolaria.

Mortensen (1931), after his discovery of the brachiolaria in *Archaster* and *Acanthaster*, restated his views as follows:

We know now the Brachiolaria to be the more generally occurring type, the true Bipinnaria being known to occur only in *Astropecten* and *Luidia*, whereas the Brachiolaria is known to occur in *Archaster*, *Acanthaster*, *Asterina*, *Porania*, *Asterias*, as also the *Solaster* larva must be regarded as a reduced Brachiolaria. This fact might perhaps speak for the Brachiolaria being the original type, as is the opinion of MacBride. Still, the fact that all the larvae pass through a typical Bipinnaria stage, before reaching the Brachiolaria stage, is decidedly in favour of regarding the more simple Bipinnaria as the primitive type; so too is the fact that the simple Bipinnaria is peculiar to the *Astropectinids* which are generally regarded as the more primitive type of Asteroids.

One fact appears certain. Larval evolution has occurred to a considerable degree in the Asteroidea, though not along channels markedly correlated with adult taxonomy. Mortensen's contention that phylogenetic speculations based on the larval structure are unjustified appears to be the inevitable conclusion.

III. DIRECT DEVELOPMENT IN ECHINODERMS

1. *Incidence*

Until recently it had come to be generally assumed that all typical echinoderms have an indirect mode of development; whenever an echinoderm had been found to depart from this criterion it had commonly been glossed over as atypical. In 1945 I drew attention to the fact that this belief is not supported by available evidence. On the contrary, a survey of the known embryological data of particular faunas indicates that no special mode of development can be taken as the general rule for the phylum.

There are some sixty-one British echinoderms about which sufficient information is available to show, with a reasonable degree of certainty, the type of development followed. Of these, direct development of some kind or other probably occurs in 70% of the Holothuroidea, 63% of the Asteroidea, 25% of the Ophiuroidea, 14% of the Echinoidea, and apparently in all the Crinoidea. Among the New Zealand Ophiuroidea, 63% of the species about which any embryological knowledge is available probably have a more or less direct development. Of the Antarctic ophiuroids studied by Mortensen (1936), there are some fifty-six species whose development is known in part, or which can be deduced with probability, and 70% of these appear to have a direct development. No doubt similar evidence would be provided by other faunas. It is clear that the notion that echinoderms normally have an indirect development, with pelagic larval stages, cannot be maintained. Rather, it would appear that the particular kind of development followed depends on particular conditions obtaining in each species—conditions which can be analysed with some measure of success.

2. *The origin of direct development*

In my opinion the causes of direct development are closely related to the physical characters of the ovum (Fell, 1945). The Ophiuroidea can conveniently be grouped into three major categories according to the nature of their ontogeny. These categories, of course, have no phylogenetic significance, and cut across natural taxonomic groupings.

Group 1 includes ophiuroids with small eggs (not greater than 100 μ in diameter), such as *Ophiothrix* and *Ophiocomina*. The egg is deficient in yolk and the cytoplasm is not abundant. They have the common character of undergoing a long, indirect mode of development, involving a well-formed pelagic ophiopluteus, followed by a pronounced metamorphosis. Group 2 includes ophiuroids with eggs of intermediate size (between 100 and 300 μ approximately), and in which there is a moderate quantity of yolk material. The yolk (as also the cytoplasm) can be measured by fairly accurate means, and in a typical case is equal to $8{\cdot}8 \times 10^4$ cu.μ. Examples are *Amphipholis squamata* and *Ophioderma brevispina*. These exhibit intermediate stages in the nature of the development, which involves a non-pelagic non-feeding larva of a variable degree of simplicity, sometimes vestigial. Group 3 includes ophiuroids with large, yolky eggs (from 400 μ upwards). Here the yolk and the cytoplasm are abundant, the yolk in a typical case measuring $5{\cdot}3 \times 10^6$ cu.μ, thus exceeding that of Group 2 by some sixty times. The cytoplasm also reaches the relatively high value of 92% of the volume of the entire egg. The mode of development is absolutely direct, without trace of a larval stage. Typical species are *Ophiomyxa brevirima*, and Kirk's ophiuroid (the specific identity of the parental form being yet in doubt). Thus with increasing egg size there is associated a steadily increasing tendency to undergo direct development.

The increase in yolk does not greatly modify the process of cleavage, as segmentation in most forms is total. In *Amphiura vivipara*, however, according to Mortensen (1921), a blastoderm forms on the animal hemisphere. In all cases where yolk is abundant there is at least a tendency to form micromeres and macromeres. Embryos of Group 1 have a symmetrical blastula with a large central blastocoel. In Group 2 the wall of the blastula is thick and the blastocoel tends to be mainly in the animal hemisphere. In Group 3 the blastocoel is reduced to a vestigial meniscus-shaped cavity in the animal hemisphere, while the wall of the blastula is greatly thickened by yolk-laden cells arranged compactly; the wall is several cells thick. The mesenchyme fails to separate as such, but remains as a great bulging mass projecting upwards into the blastocoel. The reduction of the blastocoel has a profound effect on gastrulation.

In the non-yolky embryos of Group 1 gastrulation is effected by invagination from the vegetal pole. The result of yolk in the other groups is, first, to reduce invagination to a solid inpushing of mesenchyme cells; secondly, to bring about a subsequent immigration of micromeres to contribute to the mesendoderm. The archenteron in Group 3 is thus vestigial; it disappears, giving rise to no definitive structure. The enteron is later excavated by a process of splitting in the mesendoderm. In this case the vestige of the blastopore becomes the mouth, whereas in Group 1 it gives rise to the anus. Thus the distinction between mouth and anus in regard to their mode of development is not a significant one.

At this point in development there is a still wider parting of the ways in the various groups. The heavily-yolked embryos proceed to adopt radial symmetry, whereas the others become bilaterally symmetrical. The apparent hiatus between the two categories, is, however, illusory, as is apparent from the following considerations.

3. *Recession of metamorphosis and the loss of larval form*

To account for the change from indirect to direct development, with consequent elimination of the bilaterally symmetrical stage, I have suggested that with an increasing yolk mass there has been a tendency for a backward shift in time of the point at which radial symmetry is adopted, a concept termed 'recession of metamorphosis' (Fell, 1945). The essential features of this hypothetical process are illustrated in Fig. 6 (it should be noted that the blastula and gastrula of *Ophiopluteus claparèdei* are still unknown, so that their nature has had to be deduced by interpolation). It is envisaged that with the evolution of yolk the backward shift of the time of metamorphosis has operated so as to remove from development in succession one ontogenetic stage after another, with an end-point in forms where the gastrula itself 'metamorphoses' into a radial form.

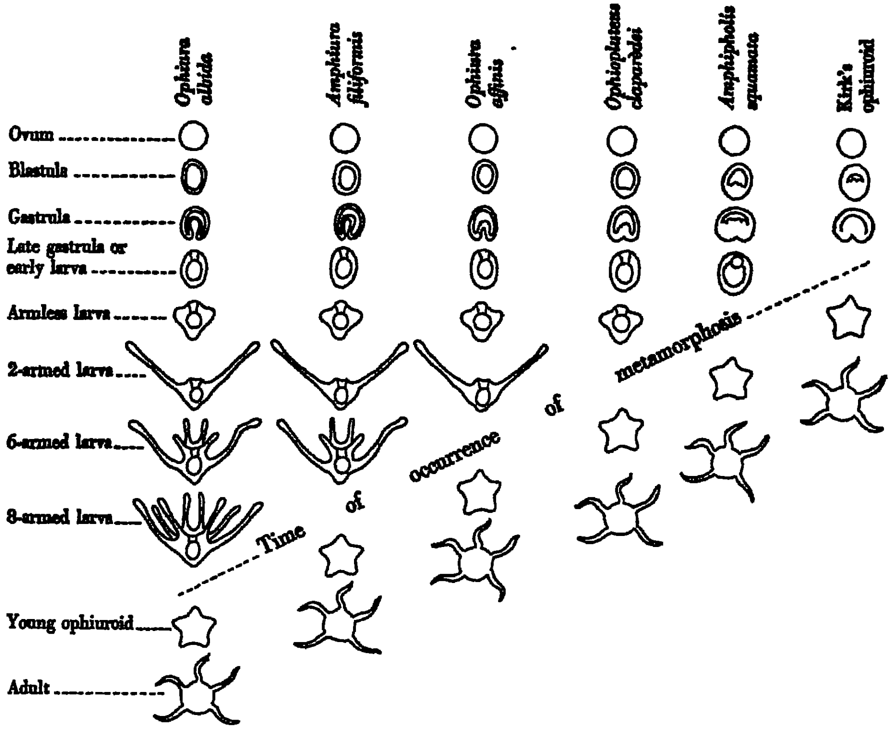

Fig. 6. Diagram illustrating the theory that metamorphosis in ophiuroids has tended to undergo a backward recession towards the gastrula stage.

If this theory of receding metamorphoses be true, it is reasonable to expect in nature a bioseries illustrating transitional stages. A survey of ophiuroids shows that such does in fact exist, and no doubt similar bioseries will be found in the other echinoderm classes.

Fig. 6 shows a series of adult ophioplutei, i.e. larvae which have reached the point in development at which metamorphosis is imminent. In the fully developed ophiopluteus, as exemplified by *Ophiura albida*, the full complement of four pairs of larval arms is present, each supported by a skeletal rod. There is a functional alimentary canal, with stomodaeal oesophagus and proctodaeal intestine leading to the mouth and anus respectively. *Amphiura filiformis* has an ophiopluteus in which the posterodorsal arms have disappeared, and the postoral pair have become reduced in size. Other features are unaltered. *Ophiura affinis* has completely lost both the postoral and anterolateral arms, as well as the posterodorsal pair. Thus there remain only the posterolateral pair. In the yolk-bearing *Ophiopluteus claparèdei* all the arms have disappeared, and are represented evidently by ciliated 'shoulders' on either side. A mouth is present but apparently the anus has been lost. In *Amphipholis squamata* the larva is no longer pelagic, being a vestigial stage in a viviparous species. The only trace of the arms is found in two skeletal rods, the mouth and anus are absent, though a vestigial alimentary canal is present. In Kirk's ophiuroid and *Amphiura vivipara* the larval stage has vanished. We are really witnessing a process of neoteny in this sequence, the larval forms becoming 'adult' at successively earlier stages of development.

There are reasons for regarding the bioseries as a regression and not as a progression. It is improbable that skeletal rods of the arms would arise earlier than the arms themselves. Therefore the existence of skeletal rods in *Ophiopluteus claparèdei* and *Amphipholis squamata* indicates the vestigial, not primitive, nature of these forms. A closed non-functional alimentary canal, as in *Amphipholis*, must be a vestigial condition. Finally, since the simplified larvae, and the species which lack larvae, develop from yolky eggs, their secondary nature is evident; yolk is unlikely to be a primitive feature.

4. *The coelom in ontogeny*

It had long been supposed that the coelom of all echinoderms is an enterocoel; one, that is, which arises from paired pouches nipped off from the archenteron. There can now be no doubt that a schizocoel is often present in echinoderms, and probably nearly always occurs in forms with yolky eggs. The various modes of development of this complex system of cavities lined by epithelium may be very briefly summarized by reference to the three main ontogenetic groups of ophiuroids.

In *Ophiothrix*, representing Group 1, MacBride records that right and left enterocoels form. The left divides into anterior and posterior parts, and soon afterwards the right does so also. From the posterior wall of the left anterior coelom the hydrocoel arises. The wall of the left posterior coelom forms the arms, and its wall the general perivisceral coelom. The right coeloms become vestigial. Other complexities reported by MacBride have since been stated to be a misinterpretation (Narasimhamurti, 1933).

In *Amphipholis*, representing Group 2, I have recorded structures in the vestigial larva which can be homologized with right and left enterocoels, but these arise by schizocoelous splitting within solid masses of mesendoderm on either side of the enteron (Fell, 1946). Occasionally a small posterior pouch is found. The right pouch, and the posterior one if present, soon disappear, and contribute to the general mesoderm, but the left pouch survives to form the five-lobed hydrocoel in the usual way. The general perivisceral coelom and other accessory parts of the coelom, all arise much later in the radial form by schizocoelous splitting in mesenchyme. In Group 3, represented by Kirk's ophiuroid, the entire coelom is formed by splitting in mesenchyme (Fell, 1941). There is a period when the free-living young ophiuroid has no general body cavity, as the perivisceral coelom forms quite late in development. A similar condition appears to hold in *Ophiomyxa brevirima*.

5. *Causes of direct development*

It may be regarded as reasonably proven that the accumulation of yolk and cytoplasm in the egg is in some way responsible for suppression of the larval form. The salient features of the sequence of reduced larvae studied above are: first, a progressive reduction in size and number of the paired larval arms, followed by loss of the arm skeleton; secondly, an increase in the amount and importance of mesenchyme, within which ultimately the coelom is excavated in place of coelomic pouches from the enteron; finally, there is complete loss of bilateral symmetry. The order in which these reductions occur, in terms of increasing yolk mass, suggest progressive inhibitions in metabolism along the axes of a bilaterally symmetrical body.

Child in 1916 showed that a wide range of simple chemical substances could cause an inhibitory effect very similar to those noted above. He was able to produce echinoplutei showing successive degrees of reduction and obliteration of antero-posterior, medio-lateral and apico-posterior differences through inhibitions of the axial metabolic gradients. In extreme cases his larvae resembled the peculiar armless larvae which we now know to develop from yolky eggs. Child drew the inference that echinoderm larvae may have been evolved by increases in metabolism along the metabolic gradients, producing the outgrowth of paired arms, etc. I have suggested that a reversal of the process would account for the regression series, and that the associated yolk material may have been the inhibitory agent (Fell, 1945). It must be conceded, however, that the apparent chemically inert character of yolk material makes it improbable that it could act in a manner strictly comparable with a simpler substance. There are further parallels. Child found that mesenchyme was unaffected by the inhibitory influences, and underwent a great increase at the expense of the bilateral organs. The same increase in mesenchyme is seen in relation to loss of bilateral organs in embryos derived from yolky eggs. Successive loss of bilateral organs implies an effective shift of metamorphosis towards the gastrula stage; the bilateral organs—the essential features of an echinoderm larva—gradually cease to intervene between the gastrula and the final form.

6. *Phylogenetic implications of direct development*

The phenomena associated with direct development serve yet again to emphasize the unreliability of larval forms as evidence of phylogenetic relationships. In these cases evolution has affected the larva so as to bring about its degeneration and eventual disappearance, while the adults retain all the features of echinoderms quite unaltered. The coelom of an adult *Ophiomyxa* shows no significant difference from that of an adult *Ophiothrix*, yet the modes of origin of the structure are entirely different in the two cases. The coelom is evidently subject to severe alterations in its mode of development. In deducing supposed relationships between hemichordates and echinoderms, certain similarities between the embryonic coelom of selected larvae have been much stressed as important evidence. When it is considered how variable the coelom can be in regard to its embryological origin and initial form, the validity of any phylogenetic importance attached to its ontogeny becomes extremely dubious.

IV. VIVIPARITY IN ECHINODERMS

Viviparity is of interest in echinoderm reproduction owing to the modifications of development which usually accompany its occurrence. It is commonly the case that eggs of viviparous species are large and yolky. The relationship between the parent and young is often extremely intimate, with marsupia forming in the mother, an embryonic attachment developing, and even nutritive substances sometimes being supplied to the embryo by the parent. Larval stages are vestigial or suppressed altogether.

Among asteroids simple brood-protection is illustrated by *Calvasterias suteri*, known from the sub-antarctic islands of New Zealand. The eggs are laid, and the numerous young are carried, in a large cluster about the mouth, apparently in the later stages living as commensals. In the astropectinid species *Leptoptychaster kerguelensis* Sladen (1889) reports that the young stars are hatched in the oviducts and later adhere to the re-entrant angles between the rays of the parent. *Leptasterias groenlandica* is reported by Lieberkind (1920) to hatch its young in its stomach, a very surprising circumstance with interesting physiological possibilities. In species of *Echinaster* the young are carried in the ambulacral grooves with the sides of the arms folded over them. More complex conditions are seen in *Pteraster* and *Hymenaster* where there occurs a complex dorsal marsupium, held up by modified paxillae, and opening dorsally by a special osculum. A marsupium is known also in *Granaster nutrix*. In the starfish *Asterias spirabilis* the embryo is joined to the parent by an attachment.

In ophiuroids all degrees of brood protection and viviparity are found; the condition is very common in antarctic species, of which Mortensen (1936) has recorded thirty-one as viviparous. A very interesting case has been recorded in Britain by Smith (1938) who found numerous young individuals of *Ophiothrix fragilis* clinging to the spines and tube-feet of adults, larger young ones occurring separately in the parental bursae. As this species has non-yolky eggs and well-developed pelagic larvae, this is an unexpected case and indicates how viviparity might arise in such forms. Smith regards the case as equivalent to natural crevice sheltering. In truly viviparous ophiuroids the eggs are usually large, and either many young may be found in the bursa, as in the New Zealand species *Ophiomyxa brevirima* (Mortensen, 1924; Fell, 1941*a*); or several, as in *Pectinura cylindrica* (Mortensen, 1924; Fell, 1941*a*); or only a few, as in *Amphipholis squamata* (Fell, 1946). The last-mentioned species, as others of the same genus, is notable in having an embryonic attachment to the parent. This structure is not nutritive; reasons have been given for believing that a nutritive fluid is secreted by the bursal wall, a conclusion supported by experimental culture of embryos *in vitro*, when it is found that the latter require chemical additives in order to develop normally (Fell, 1940*b*).

In comatulid crinoids, where the eggs are large and rich in yolk, it is the general rule for the young to adhere to and develop on the pinnules of the parent. In some species, however, the eggs are free, as for example *Tropiometra carinata* and *Antedon petasus* (Mortensen, 1921). True viviparity, involving special marsupia, is found in antarctic crinoids such as *Isometra vivipara* (Andersson, 1905; Mortensen, 1920), and *Thaumatometra nutrix*. In *Notocrinus virilis*, also, Mortensen (1921) records marsupia, and it appears that in this species the young are nourished by an albuminous secretion from the wall of the marsupium.

Similar degrees of brood protection and of viviparity are to be observed in the holothurians, where the incidence in antarctic species is as pronounced as in the other classes. In the simplest type the embryos merely rest on the surface of the body. In other species small alveoli may form in the integument, enclosing the eggs. Sometimes the eggs develop within the oviducts, or within the coelom. In other cases an invagination or evagination of body wall will form a marsupium. Genera showing viviparity include *Cucumaria*, *Thyone*, *Psolus* and *Phyllophorus* among Cucumariidae, and *Chiridota*, *Synaptula* and *Leptosynapta* among Synaptidae.

Since echinoids are distinguished from other echinoderms by possessing generally small eggs and pelagic larvae, viviparity is necessarily of limited incidence in the class. Among cidarids Mortensen (1926) has shown that the New Zealand *Goniocidaris umbraculum* carries the young in the partly sunken peristome, covered over by oral spines, thus forming a marsupium. In other cidarids the spines of the apical part of the test form a marsupium. Among the spatangoids the sunken petals frequently serve as marsupia, as in *Schizaster*, *Hemiaster* and others. In some species sexual dimorphism of the test is a consequence of this condition.

It is clear that brood protection and viviparity can influence development considerably. Larval stages can be effaced or at least reduced. New embryonic organs may evolve, such as the attachment stalks between embryo and parent. New modes of nutrition may arise, involving a change from ingestion of planktonic food in a functional alimentary canal to surface absorption of fluid material by the outer epithelia of the embryo. Yet in these cases the end result is the same—an adult echinoderm arises quite as typical of its group as an oviparous form. Here, then, is just one more illustration of the potency of evolution in acting upon embryonic stages without significantly affecting the adult.

V. ECHINODERM EMBRYOLOGY AND THE ORIGIN OF CHORDATES

The foregoing survey has attempted to collect together the salient features of echinoderm embryology which can have any bearing on the phylogenetic interrelationships of the several classes within the phylum, as well as on the wider problem of whether there is any relationship between the echinoderm and chordate phyla. The conclusion appears to be inevitable that intolerable discrepancy exists between phylogenetic inferences drawn on the basis of the recapitulation theory and those drawn from comparative morphology and palaeontology. Each of the two opposed sources of evidence, if utilized to construct a hypothetical phylogenetic tree, provides a result absurdly different from the other. In illustration, let it be assumed that both approaches to the problem are legitimate. The two contrasting results will then be reached on lines somewhat as follow.

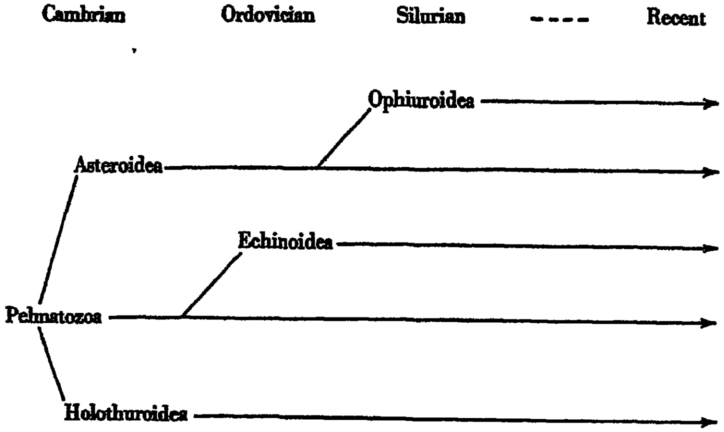

Fig. 7. Apparent relationships of echinoderm classes as suggested by morphological and palaeontological evidence.

Fig. 7, while in no way intended to represent established fact, indicates very approximately the kind of phylogenetic relationships between the extant classes of echinoderms and fossil species which morphology and palaeontology suggest. Already in Cambrian times Pelmatozoa, Holothuroidea and Asteroidea had become distinct groups. Morphology suggests that the two latter arose from the Pelmatozoa. Echinoidea, which appear in the Ordovician, seem to have arisen directly from Pelmatozoa. Ophiuroidea appear in the Silurian, and fossil evidence leaves little doubt that they arose from the asteroid stem—a conclusion which comparative morphology also supports. There is no fossil evidence to suggest any connexion between echinoderms and the chordate stem.

Fig. 8 represents the kind of phylogenetic tree which the recapitulation theory would construct from embryological evidence, if larval forms are regarded as repeating ancestral conditions. A common dipleurula ancestor gave rise to three stems. The first led to the Pelmatozoa, the other two led to a pluteus and an auricularia respectively. Later the pluteus ancestor gave rise to ophiopluteus and echinopluteus forms, and these led to the Ophiuroidea and Echinoidea respectively. The other line, the auricularia, gave rise to three forms. One of these, a pentacula, led to the Holothuroidea. Another was the bipinnaria, which led to Asteroidea, with a possible anchored brachiolaria ancestor intervening in some groups as a recapitulation of the original Pelmatozoan stalked stage. The remaining descendant was a tornaria, the ancestor of the Hemichordata, and, by assumption, of all other chordates. The vitellaria larva, the pupa of crinoids, and the other larval forms fail to fall in line with the foregoing, and have to be relegated to the status of 'caenogenetic structures'.

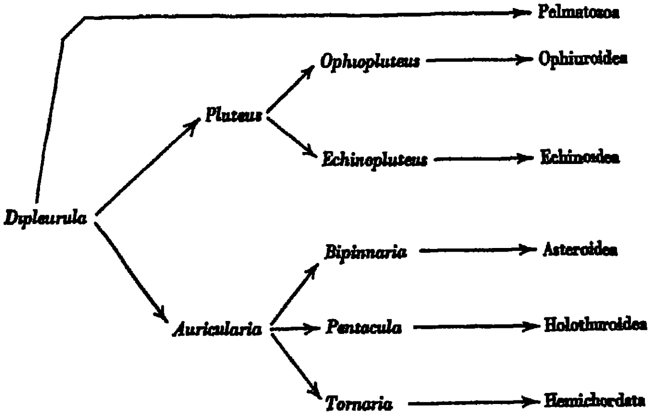

Fig. 8. Apparent embryological relationships of echinoderms and hemichordates.

The second account, based on the supposition that larval forms retain ancestral structure, is grossly opposed to what all other evidence would lead us to believe are the relationships between echinoderm classes. It is impossible to accept the result which implies that ophiuroids and echinoids are more closely related to each other than to the other classes, and that holothurians and starfish are similarly connected. If, therefore, the recapitulation theory as applied to larval forms leads to a *reductio ad absurdum* in the case of echinoderms alone, the result can only be regarded as equally unsatisfactory in regard to the supposed chordate connexion, for which there is no other palaeontological or morphological evidence.

Manifestly, evidence from these larval stages is not susceptible to inductive reasoning on the basis of their being recapitulated ancestral stages. The reason for this has become clear now that the extremely variable nature of the echinoderm larva is known to be one of its chief attributes. The position appears to be analogous to the *impasse* arrived at some years ago in connexion with the taxonomy of digenetic trematodes. Widely divergent phylogenies were proposed by various workers, according to whichever particular larval characters were regarded as significant. Later, however, the stability of the excretory organs was established, and a more satisfactory classification developed. In the echinoderm larvae there seems to be no comparably static larval organ. The nearest approximation is found in the ciliated band, but even this is stable only during the initial dipleurula stage.

This survey has shown how, in each of the various fields chosen for particular examination, variation of the larval form can occur, often resulting in wide divergence within small groups, and equally often resulting in surprising convergence between distantly related groups. Since larval variation of a profound extent can be observed in particular cases, it becomes highly probable that the same is true of other cases where the point cannot yet be proven so clearly. Thus there is every reason to accept Mortensen's view that the characteristic echinoderm larval forms are specialized stages, without phylogenetic significance in any but a very limited extent. Consequently, since we cannot interpret resemblance between the auricularia and the bipinnaria as indicating any close relationship between the two classes which possess these larvae, neither can we attach any greater importance to the resemblance between the same auricularia and the tornaria of hemichordates.

It is therefore obligatory to draw the conclusion that hemichordates do not exhibit any significant relationship with echinoderms. Since no other chordates—supposing that hemichordates are chordates—resemble echinoderms, the whole assumed relationship between the two phyla now rests upon some biochemical evidence of a slender character.

In reaching this conclusion the evidence supplied by echinoderms has not been the only source of information. It is necessary to recognize that very little is known as yet of the embryology of hemichordates. The tornaria larva is one about which no wide body of data is available; comparative embryology does not yet exist within the hemichordates in the sense that it does in echinoderms. Not all hemichordates possess a tornaria. Although the complete embryology of the New Zealand *Dolichoglossus otagoensis* has not yet been worked out, it is certain that no tornaria stage occurs in its life history. The embryo proceeds to adopt the adult form without any pelagic larval stage (Kirk, 1939). It is therefore clear that evolution has operated upon the embryonic stages of at least one hemichordate in much the same manner as it has in echinoderms.

Clark (1937) has been forced to the conclusion that the various animal phyla arose independently. In regard to the topic here discussed he writes (private communication, 1945): 'The whole subject is complicated by the undependability of the evidence afforded by the larval stages. We need to know far more about the ecology, so to speak, of larvae than we do now. Larvae seem to be as much subject to adaptation to their surroundings as are the adults, at least in many groups....There is no doubt that the echinoderms are all closely related; but the relationship is to be sought only in the very early stages, no later than the late gastrula. Thus I do not believe that the vertebrates were derived through the echinoderms'. Although perhaps a majority of workers is still inclined to regard the dipleurula larva as recapitulating a common ancestral form of existing echinoderms, in his main conclusion Clark receives strong support from the facts now known about echinoderm embryology.

VI. SUMMARY

1. Development in echinoderms may be indirect, involving pelagic, bilaterally symmetrical, larval forms, or more or less direct, with the larval stage either reduced or omitted. Of the five living classes, only the Echinoidea are characterized by being predominantly of the type with indirect development.

2. It is possible to regard the dipleurula stage of the classes Asteroidea, Ophiuroidea, Echinoidea and Holothuroidea as recapitulating a common ancestral *Dipleurula*. It is no longer possible to regard any of the other types of echinoderm larvae as anything but specialized forms without broad phylogenetic significance.

3. Embryos and larvae of echinoderms are extremely plastic, often exhibiting convergence, divergence and adaptation, susceptible to evolutionary modifications of structure which may act quite independently of the adult stage. Ancestral structure cannot be deduced from such forms.

4. In the Echinoidea larval evolution seems to have occurred subsequently to the separation of the main orders and families. Within relatively small groups larval evolution has followed similar trends, so that characteristic larvae occur in various sub-groups, where the young stages may follow similar ontogenies; but such independent evolution tends to obscure the phylogenetic relationships between the class as a whole and the other classes.

5. In the Asteroidea larval evolution has occurred along channels not so markedly correlated with the taxonomy of the adults. Phylogenetic speculations based on such larval stages prove incompatible with other evidence.

6. In the Holothuroidea and Ophiuroidea larval evolution cannot at present be related with adult taxonomy, save in one or two cases too unimportant to have general significance.

7. The egg of echinoderms is liable to undergo changes in volume. Increase of volume is directly related to increase in cytoplasm and its product, the yolk material. Such increases have led to direct development.

8. Increase in cytoplasm and yolk has not greatly affected the cleavage process, which is almost always total. A distinction between micromeres and macromeres frequently results.

9. With increasing cytoplasm, the wall of the blastula becomes thicker, and the blastocoel is in extreme cases reduced to a vestige in the animal hemisphere. The mesenchyme fails to separate as such, but projects as a solid mass into the blastocoel. Invagination is reduced to a solid inpushing of cells, and epiboly may ensue. The archenteron may become vestigial, in which case the definitive enteron is excavated in the solid endoderm by splitting. The enterocoele become reduced or lost, and the coelom and its adjuncts may arise by schizocoelous splitting in mesenchyme.

10. In Ophiuroidea a succession of stages in reduction of the ophiopluteus may be seen, suggesting a recession backwards in time of the moment at which metamorphosis is initiated. In extreme cases the gastrula itself becomes radially symmetrical and the larva is completely lost.

11. By convergent evolution among echinoderms with yolky eggs, a special vitellaria larva has arisen independently in Holothuroidea, Ophiuroidea and Crinoidea. The

vitellaria is characterized by its barrel shape, and the transmutation of the ciliated band into annuli. In the Crinoidea this is the only larva as yet known.

12. Viviparity does not seem to have been an important factor in causing direct development, though it may influence the physiology and morphology of the young stages.

13. If larval stages of echinoderms are interpreted as recapitulating ancestral stages, the conclusions reached are seriously discordant with other evidence. Therefore it is not possible to base phylogenetic interpretations on larval stages alone.

14. Echinoderm embryology cannot provide any valid support for the hypothesis that chordates arose from echinoderms.

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AERIAL PLANKTON AND ITS CONDITIONS OF LIFE

By TORSTEN GISLÉN
University of Lund, Sweden

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I. INTRODUCTION

Organisms measuring more than a few millimetres in length have distributional areas which are always clearly circumscribed from the geographical point of view. The cause of this, as a rule, is historical: a particular species had possibilities in a previous epoch of spreading between the areas now isolated from one another. Thus most of the larger Swedish terrestrial animals arrived in Sweden during the *Ancylus* period, seven to nine thousand years ago, when a land bridge over the Danish islands united Sweden to north-west Germany. In a similar way the Caspian Sea probably received part of its marine fauna during a certain part of the Quaternary period, when this sea communicated with the North Polar basin. Evidently the cause of this geographically well-circumscribed distribution of larger organisms is due to barriers which cannot be surmounted under usual conditions. This does not always mean that the particular organisms are unable to thrive in areas from which they are now absent. Plants and animals introduced by human agency have in some cases easily adapted themselves to new surroundings and increased with explosive rapidity, as for instance the sparrow in North America, the rabbit in Australia, the mitten crab (*Eriocheir sinensis*) in the rivers of western Germany, or the Canadian pond weed (*Elodea canadensis*) in Europe.

Turning, on the other hand, to small forms and micro-organisms below a few millimetres in length, detailed investigations have shown that distributional limits of the type mentioned above for the larger forms are of much less importance or do not exist at all (Gislén, 1940). In fact, their distribution tends to be more or less cosmopolitan. It is true that they are often restricted to a certain climatic belt, and within this they are as a rule strictly bound to certain ecological surroundings corresponding to their needs. But where these needs are realized—and this can be the case in a vast number of places, as their demands for living space are very restricted—they appear everywhere on the globe, if only the time factor has been adequate. This applies both to terrestrial and aquatic organisms.

Such a cosmopolitan distribution is perhaps not so remarkable in the case of small marine organisms, where currents facilitate a wide distribution of the planktonic larval forms. Quite recently, geologically speaking, there was a free communication around the globe between the various tropical oceans, thanks to the circumtropical Tethys Sea. In this way there was until the middle part of the Tertiary period a rather uniform tropical marine fauna and flora. Since the separation of the oceans at about the end of Miocene times different parallel forms have, in most cases, developed in the various seas. There are forms, however, especially among planktonic but even among benthic animals, which still retain their specific identity on both sides of continental or oceanic barriers, in this way demonstrating the very slow rate of evolution of marine organisms (Gislén, 1944).

Marine forms, especially planktonic ones, have possibilities of spreading in a vast, more or less uniform medium. Because of this and of their conservatism in evolution they are widely distributed. Entirely different problems appear when we turn to terrestrial or limnetic organisms. Fresh-water forms can only spread directly as far as the limits of the water system in which they occur. Terrestrial forms may be impeded in their distribution by water, mountain ranges or deserts, all of which provide barriers which may be totally impassable to larger forms.

As mentioned above, these obstacles are absent in the case of small organisms. For these the only limiting boundaries are climatic and edaphic. Therefore, wherever surroundings are suitable the most astonishing finds can be made of forms which may have been described earlier in far away places on the globe. Thus, among the Mycetozoa several species have been reported partly from Europe and North America, partly from South Africa and South Australia, and there are species known to occur on all five continents. The same is true of the Rhizopoda, Ciliata, Rotatoria, Tardigrada, Cladocera, Apterygota, and others (Gislén, 1940). Similar cases of wide distribution have been recorded for certain algae, fungi and soil bacteria (e.g. Barthel, 1923, p. 60; Lange, 1934).

How are these facts to be explained? It seems as if the increased ability to spread were caused in the first place by the influence of the wind in transporting small objects. But there are other distributional agencies, such, for example, as water or the fur and feathers of large animals. .

II. DISTRIBUTION OF LARGE ORGANISMS

Even large forms can be distributed through the air by birds. As a rule it is the reproductive stages alone, but full-grown forms too, such as leeches, are sometimes spread in this way. Neiman (1924) showed that spawn of two species of pond snails could be transported when stuck to a glass plate and hung out of the window of a train during a journey of 30–75 min. without serious reduction in the number of eggs which developed. The older the embryos the better they withstand such transport. Incidentally the experimental conditions in this instance were much less favourable than those in transportation by a bird, as in the latter case there are often protecting hollows or feather-clad areas where organisms carried on the bird can survive more readily. That long distance transport by birds can actually take place is shown by the fact that a duck was shot in the Sahara at least 100 miles from the nearest body of water with fresh mollusc spawn attached to one of its feet (Weber, 1914, p. 520).

Water currents may also be important. Palmén (1944) has found that insect species new to Finland are often driven ashore in a living state on the southern coast of that country. In this case, however, the animals, often in an exhausted condition, reach a shore which is unsuited to their mode of life. They therefore usually perish before reaching localities where they could survive and propagate. In spite, therefore, of living specimens of new species being driven ashore every year, these do not become indigenous.

As regards distribution by air, there was considerable early speculation. Only recently, however, has it been possible to obtain a tolerably correct conception of the appearance and frequency of the organisms present in the air. It is now certain, however, that the air harbours an invasion army, rich in small animals, plant-spores and so on, which are constantly recruited from the earth's surface. These form the plankton of the air.

In our latitudes violent winds are infrequent, but there exist, for instance on hot days, strong upward thermal convection currents. From measurements carried out by aeroplanes it is known that these upward currents can reach altitudes of at least 1000–1500 m.; they are generally not strong above 300 m. At night the air cools down, especially near the earth's surface, and especially in the lower air layers. There is then a tendency to develop downward currents. The higher layers of air are cooled more slowly than those near the ground and will, therefore, be a little warmer at night. Upward currents are also produced when a horizontal wind meets a mountain range. The wind passes over the range, often with great velocity, and then diminishes in force. By different degrees of heating of the air, for instance over land and over water, differences are produced in the upward currents within a given area. Clouds too play an important part in the intensity of upward movements of the air. Over smaller heated areas air pockets may be found. Strong air currents also appear around a storm centre and in connexion with heavy rains.

In hotter countries the air may, on certain occasions, be subjected to exceptionally violent movements. Tornadoes and typhoons at their maximum strength can produce a wind velocity estimated to be about 100–250 m./sec. With such a velocity the tempest can carry with it all that comes in its way. McAttee (1917) made an inventory of some singular cases. Thus an iron screw weighing 338 kg. was hurled 270 m., a chicken coop of 4 × 4 ft. and a weight of 38 kg. was transported a distance of 7 km., a tin roof went 25 km., and a church spire 28 km., before reaching terra firma again.

A spout formed by a tornado has the power of sucking up water. In this way small fishes, invertebrates, and small loose plants will make an occasional journey through the air before they reach the earth's surface anew. These so-called rains of fishes have most often been reported from India and have been witnessed by scientists. Gudger (1921, 1929) related a number of interesting cases. De Castelnau (1861) reported from Singapore that he found masses of the fish *Clarias batrachus* in his garden after an exceptionally heavy rainstorm. In Katywar there was a colossal downpour of rain in the year 1850; in only 90 min. 185 mm. of rain were registered, and in this time so many fish fell from the air that the earth was literally covered with them. Rains of fish have also been reported from more temperate zones, namely New York, Holland, the surroundings of Paris, and Great Britain. In Scotland small herring rained down 3 miles from the sea coast. Further data are given in the papers of Gudger referred to above. Fishes transported through the air rarely measure more than 5–10 cm. in length, but some are up to 15 cm. (exceptionally 30 cm.). Sometimes the upward, often very humid, air currents rise to such altitudes that the moisture carried with them may freeze and be precipitated in the form of great hailstones. In exceptional cases small frozen fishes have been found inside these hailstones, and in one case, in America, even a small turtle.

An important fact is that in several of the cases mentioned the transported animals were still alive. Prévost threw small living fish from the roof of a house 30 m. high down on to wet mud and found that they survived. The experiments were continued by dropping small trout from an aeroplane on to frames with netting floating in a lake. The fish could thus be recaptured and it was found that all of those that had fallen within the frames were in good condition, provided only that they were less than 7·5–12·5 cm. in length. With this small size the resistance of the air is so large relative to the weight of the falling body that a constant, rather moderate velocity is acquired which does not endanger the life of the animal when it hits the water. The trout was generally dropped from a height of 100 m., but above an altitude of 25 m. the speed of falling was in fact not increased. The method has been used in Canada to implant trout in lakes which have been devoid of fishes since the Glacial Period (Prévost, 1935, and in a letter; Prévost & Piche, 1939; Darlington, 1938*a*, p. 282).

Although wind may transport even vertebrate animals through the air, yet this method probably does not play a large part in the distribution of these animals. Nevertheless, Gulick considers (1932) that the absence of salamanders, frogs, larger molluscs, and mammals on an island is evidence against a land bridge to the island. Darlington (1938*a*, p. 283) has pointed out the possibility of an aerial dispersal of even relatively large animals by huge palm-leaves, in the rolled basal parts of which they may be transported safely during hurricanes.

III. DISTRIBUTION OF SMALL ORGANISMS

The presence of small molluscs on an island might be explained through transport by birds or by wind, for if there had been a former land bridge a larger part of the fauna of nearby areas should also have invaded the island. The fauna of many oceanic islands seems to be composed by mere chance. Darlington (1938*b*), who investigated carabid beetles occurring on Mediterranean and West Indian islands, states that they are all small and are probably driven by weather from nearby continents. By means of hurricanes, for instance along the chain of the Antilles, the aerial transport of small forms might be expected to occur regularly. But weaker wind currents may also act as dispersers. Regular migrations of insects are known from North America. In the spring they come northwards with northerly winds, in the autumn southwards with southerly winds. Gypsy moth larvae have been shown to be transported by wind for distances of 32–50 km. (Collins, 1917).

Investigations have been carried out with catching apparatus, transported in the air and exposed at a certain height, later to be closed before return to the ground. Such experiments have been made both with kites (Hardy & Milne, 1937, 1938) and from aeroplanes. These investigations were begun during the 'twenties, and preliminary results were published in the beginning of the 'thirties by Coad (1931) and Berland (1935). In 1939 Glick published a comprehensive paper on the subject in which he analysed the results obtained by an examination of his large amount of material. In these investigations it was shown that up to considerable heights there exists an important aerial plankton. Berland investigated the air up to 2300 m. in the vicinity of Paris. Glick reported flights carried out in Louisiana between altitudes of 6 and 4500 metres throughout a year both by day and by night. During 1000 flying hours 30,000 animals were captured. In order to obtain comparable results the catching apparatus was always exposed during a flying time of 10 min. The maximum quantity of air plankton was obtained in May, the minimum in December and January. During calm days more insects were obtained at 60 m. than during windy ones; during days characterized by agitated air masses there was an increase in the layers between 300 and 1500 m. By night a somewhat larger number of animals was obtained below 600 m. than during the daytime, but higher up there was scarcely any difference. On moonlight nights rather more insects were in the air than on dark nights. With strong upward currents small animals were carried to heights of 900–1200 m. in a day. The greatest number of insects in the air was found when the sky was partially cloudy and consequently much turbulence prevailed in the atmosphere. With heavy rains and a low temperature few insects were found in the air. After rains, thanks to rising temperatures, there was much aerial plankton.

Diptera were found to be commonest; they were followed in frequency by the Coleoptera, especially Carabidae and Staphylinidae. The insects were most abundant near the ground. At a height of 60 m. the density of the population was only half of that at 6 m. At 60 m. on an average 13 specimens were obtained per 10 min. flying time; at 300 m. this figure had been reduced to 4·7. At 600 m. the number had been halved, and at 900 m. the population density was half of that at 600 m. From 900 up to 4200 m. the quantity was roughly the same. Large and strong flying insects were obtained at lower altitudes, rarely over 900 m. At higher levels the animals scarcely ever exceeded a length of 3–4 mm. (Berland, 1935, p. 90). Within any group of flying insects the smallest were always obtained at the greatest heights; often, however, they had large floating structures. Several Homoptera, Hymenoptera and Diptera were collected up to a height of 4200 m. Many absolutely wingless forms were also taken at high altitudes, and gossamer occurred up to 3300 m., one spider being even found at 4500 m. Mites and wingless ants occurred at 900 and 1200 m., and a flea was taken at 60 m. The air plankton at greater heights consists mostly of small flies, mosquitoes, small Coleoptera and Heteroptera, Homoptera, parasitic Hymenoptera, and small spiders. It has been calculated that an air column with a height of 4200 m. and a cross-section of a square mile generally harbours about 25 millions of such small animals (Hardy & Milne, 1938).

The strongly flying insects, which are found at low levels, distribute themselves chiefly by their own power of flight, while smaller forms are often swept into the upward currents and are then distributed passively. Small spiders, for example Linyphiidae, have made this wind transport their normal type of distribution. In fine late summer weather with weak air currents they spin a long thread from some elevated position; the wind seizes it, and, attached to the thread, they float up into the air.

When a storm is approaching, animals often become agitated and uneasy. With this type of weather strong gusts of wind or whirlwinds occur, which carry up small particles and small animals from the ground. A proof that most animal forms which appear at high levels in the air consist of individuals often carried from elsewhere was given during the great Mississippi flood of 1927. In flights at a height of 60 m., eight times as many individuals were found in the air plankton over the non-flooded areas than over the flooded territory. On the other hand, at an altitude of 300–1500 m. this difference disappeared and the same number of animals was obtained over land and water; the air plankton had evidently been blown in over the flooded areas.

During the journey of animals upwards through the air their existence is endangered by drought, frost, low air pressure and strong solar radiation. Many individuals die, but there are numerous cases showing that they can survive journeys at considerable altitudes and of long duration.

Hardy & Milne (1937) captured several hundred insects by kites sent up to a height of 60–120 m. over the North Sea, 200–250 km. away from the nearest land. At 215 km. off the west coast of Africa the *Discovery* was invaded by numerous moths and countless grasshoppers, locusts and flying bugs. The same vessel, 515 km. off Portugal and 570 km. off Morocco, was boarded by sphingids and even a butterfly. The large American monarch butterfly (*Danaus plexippus*), famous for its migrations from North to South America, has also been found occasionally in Ireland, England and Portugal, and even in the Southern Pacific, 830 km. from the nearest land (Williams, 1930). A swarm of migratory locusts boarded a vessel between Bordeaux and Boston, 2500 km. from the nearest land (Glick, 1939). All the insects mentioned here are larger forms which can fly far from land by their own wing-power. The wind also plays a certain part, as is shown by the devastating swarms of migratory grasshoppers which often accompany special winds and disappear when these change.

The occurrence of small forms in the catches shows that these can endure long air journeys, having been passively transported. A large number of the animal forms found in aeroplane traps are still alive when taken out. Living insects have been collected in traps after flights from 60 up to 2700 m. The following case is probably the most remarkable example of the air transport of small animals for a long distance. An expedition sent out from Oxford to Spitsbergen reported that on 8 August 1924, after a strong southerly gale, the snowfields and glaciers of North-East Land were studded with various insects, chiefly aphids. About 80% of those collected were still alive. The plant lice turned out to be *Dilachnus piceae* parasitizing the spruce. The nearest locality for spruce is on the Kola Peninsula, situated at almost 1400 km. from North-East Land* (Elton, 1925, p. 291).

It might be thought that wind would be a considerable danger to animals transported by air. A strong wind has great powers of rapid drying. Normally the velocity of the wind at a height of 1800 m. is probably more than 25 km./hr. In several cases insects have been taken in aeroplane traps at great heights, when the wind velocity was 75 km./hr. With such a velocity the transport from the Kola Peninsula to Spitsbergen would have taken less than 20 hr.; the actual force of the wind on the occasion mentioned suggests something between 12 and 24 hr. (Elton, 1925, p. 294). Floating in the air, however, the insects are not subjected to the desiccating power of the wind, since they have the same velocity. Desiccation only threatens during clear weather, while the risk is not great in a foggy or cloudy atmosphere.

Temperature, at least during the warmer part of the year, is probably not a limiting factor for distribution in the lower layers of the air. The average temperature falls about 1° C. for every 150 m. up, which means that at a temperature of 20° on the ground, freezing-point should be reached at an altitude of about 3000 m.

Air pressure at 5500 m. falls to half of that on the ground to be halved again at double that height; at 20 km. it is about 40 mm. of mercury (Lysgaard, 1943, p. 76). Lutz (1932) placed ten flies in a humid chamber in which the air pressure was lowered to 22 mm. Hg in 90 sec. Four minutes after air at atmospheric pressure had been let in again, all the flies were as active as before the test. After repeating the
expe flies were still alive. These flies, however, gave
norn rations.

M been able to verify the rather startling observations
of L hich are not yet finished, we have extended the
inves Diptera, Coleoptera, Lepidoptera, Hymenoptera,
Opil Diplopoda, Isopoda, Mollusca and Oligochaeta.
Prov were humid, the forms investigated supported

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* I node of distance distribution has arisen through air transport
by m e the strong air currents soon sweep away everything. But
vario tuffs, vegetables and flowers transported by the plane. In the
air po aterial brought in, as well as the interior of the aeroplane, is
alway this way, in 1933, insects from Central and South America
were irship *Graf Zeppelin* made its first visit to North America
in 19 d in bouquets decorating the cabins; in 1929, on a similar
occasi six of which had never before been taken in North America
(John

a lowering of barometric pressure (in 2–3 min.) to 70–50 mm. Hg before they showed signs of being seriously affected. At that pressure they sometimes defaecated, showed rather rigid movements and often became more or less extended. At a pressure of about 15 mm. most species were lying on their side or back, quite motionless and looking as if dead, but snails and oligochaetes continued to creep, probably because of the insignificant oxygen consumption for the motion of their smooth muscles. Generally the animals were kept at the low pressure mentioned for 2–3 min., after which normal pressure was restored in 1 min. Isopods, chilopods, diplopods and most spiders then began to move almost immediately, while it generally took the insects 1–3 min. before they walked and flew again. In double this time they were behaving quite normally. From this it is clear that land invertebrates have an astonishing power of sustaining and surviving low air pressures.

Back & Cotton (1926) made some experiments at low air pressures to investigate the possibility of using a vacuum as a control of insect pests. At a very low air pressure, between 8 and 33 mm., most insects succumbed in less than 7 hr. In two species, however, a small percentage of eggs and larvae were still alive after 24 hr. With a pressure varying between 10 and 85 mm. most individuals were killed in 24 hr., but a small number of eggs and larvae survived for 2 days. When the pressure was kept between 40 and 140 mm. most insects were dead after 3 days. In a few species, however, a small percentage of larvae and even adults were still alive after 6–7 days. As no arrangements were made for maintaining the humidity (cf. p. 1039), the death must have been due to desiccation. The authors state, too, that the dead specimens 'were stiff and brittle and appeared to have been thoroughly dried out'.

IV. DISTRIBUTION OF MICRO-ORGANISMS

Organisms of a size near or beneath the limit of observation with the naked eye have still greater possibilities of air transport. In investigating the powers of distribution of these forms it has not been possible to use the same methods as those applied to the larger planktonic organisms of the aerial sea. Pasteur (1860) collected dust with an *aspirateur* in order to investigate the occurrence of micro-organisms in the air. Interesting investigations were carried out by Erdtman (1937) during a journey from Europe to America. The air was on different occasions sucked up by a vacuum cleaner and the number of pollen grains per unit volume was calculated. Over the North Sea 18 pollen grains per 100 cu.m. were obtained; half way between Europe and America the figure sank to 0·7; off Nova Scotia it had increased to 3·5, and near the coast of New England it was 15. For comparison it may be mentioned that the corresponding figure over land amounted to 18,000. Pollen grains of American tree species were identified at 300 km., in some cases even as far as 650 km. from the nearest land, and pollen of some other species was found up to 1000 km. from the coast.

Charles Lindberg, however, was the first person to carry out investigations of microplankton at higher altitudes. In 1933 he flew between America and Europe, and on the way crossed the inland ice of Greenland. On the journey he used an apparatus for catching micro-organisms, the so-called sky-hook. Sterile glass microscope slides were coated in New York with a thin sticky film and were put into a sterilized tube. This was hung out during the flight. Such slides were exposed on various occasions and at different altitudes. The tubes were closed before being hauled in, and were investigated after the return to New York. The flights were carried out over the sea at an altitude of 750–1650 m., and over Greenland at 2400–3700 m. It was found that the glass slides were studded with spores of fungi, unicellular filamentous algae, spicules of sponges, insect wings, volcanic ash, etc. (Meier, 1935).

The peril of drought which threatens larger forms is still greater as organisms approach microscopic dimensions. The ratio of surface to volume increases in smaller forms. This means that such animals, if they are to make use of the possibility of distribution as aerial plankton, must possess qualifications lacking in larger animals. Some of these forms, by passing into a state of lethargy known as anabiosis can endure very considerable losses of water, in fact so great that they shrink to crackling dryness. As soon as they are wetted they imbibe water again, swell out to their original size and shape, become mobile after a quarter of an hour to an hour, and soon creep about as though nothing had happened. Such forms are found, for instance, among the Amoebozoa, among certain Nematoda and Rotatoria, and are especially prominent in the Tardigrada. All these organisms live in situations where drought threatens. Other animals, which do not themselves withstand drying up, or which do not endure transport through the air, have reproductive bodies that are exceedingly resistant.

Both the anabiotic forms and the resistant eggs can endure very long periods of desiccation (Bock, 1936; Lucks, 1929; Wülker, 1926). Eggs of rotifers, after a drought of 13–14 months, developed on being wetted again, regardless of which stage of embryonic development they were in when they dried up. Sars (1886, 1888, 1889) hatched resting eggs of Cladocera, Phyllopoda, Copepoda, and Rotatoria in Norway from mud which had been collected and dried 14–17 months earlier in tropical Australia. Dried bdelloid rotifers have revived on being wetted after 5 years' desiccation. Some species of rotifers have been allowed to freeze and dry seven times successively, after which they were still alive when placed in suitable surroundings. Nematodes living in moss have revived after 10 years' desiccation. The wheat eel-worm, injurious to agriculture, has been shown to live after lying encapsulated in its cocoon in a wheat grain for 27 years. The Phyllopoda, famous for their meteoric appearance in occasional water pools, in which they pass through their development with exceptional rapidity, have drought-resistant eggs. The eggs of many species can endure desiccation for 3–5 years, and some after drying have remained alive for 15 years (Spandl, 1925). By accommodating themselves to temporary pools, and by the exceptional resistance to desiccation of their reproductive stages, these animals have succeeded in occupying a peculiar ecological niche out of danger from modern competitors, and in this way they have been able to survive (Gislén, 1937). Encysted Infusoria may endure years of desiccation; *Peridinium cinctum* has been proved to stand a drought of over 16 years.

Bacteria do not generally withstand more than a few days of dryness. Nevertheless, there are exceptions. Spore-forming bacteria have particular possibilities of surviving in unfavourable surroundings. The malignant anthrax bacillus, which is exceedingly hardy, can remain alive, dried up on a silk thread, for 70 days; its spores can stand drought for years. A rapid drying up, as by a vacuum or in a desiccator, promotes resistance against drought rather than the contrary. The very hardy but not spore-forming *Bacillus pyocyaneus* when dried in air will not live more than 9 days, but it holds out in a vacuum for more than 7 months (Shattock & Dudgeon, 1912).

These animals and micro-organisms, however, are not only very resistant to desiccation but they also endure very varying temperatures. All the organisms mentioned here can endure higher temperatures in dry air than in humid conditions. Dry air will kill bacteria in $1\frac{1}{2}$ hr. at 100° C. Nevertheless, spores will live for 3 hr. at 140° C. Certain multicellular animals which show anabiosis during drought have turned out to be very resistant to heat as well. Dried cysts of *Colpoda* are said to resist dry heat at 100° C. for 3 days (Brues, 1939). Bdelloid rotifers withstand heat of nearly 125° C. At an air humidity of 55–65 % all organisms die at 100° C. in less than an hour. At an air humidity of 30 % bacteria perish at 70–80° C. in less than 48 hr. Humid warmth kills all spores at 120° in 30 min., and at 140° C. they die in 1 min. (Kolle, Kraus & Uhlenhuth, 1931, p. 854). As a rule, pathogenic bacteria which do not form spores die in a liquid medium at 62° C. Nevertheless, here too there are some more resistant forms. *Bacterium thermophilum* still divides at 63°, but dies in 30 min. at 71°. Certain soil bacteria are still more resistant. Globig (1888, p. 304) found that some of them thrive at 68°, and a micrococcus was observed to divide at 74°. In hot springs a flora especially rich in Cyanophyceae, has been found between 35° and 50°. Above 60° the number of existing forms decreases markedly, from seventeen species to four according to an investigation by Yoneda (1938–40). Out of seventy-seven species in the type of locality mentioned, only five survived over 64°. *Oscillatoria formosa* is known to hold out at 75° while other Cyanophyceae have been found growing at a temperature as high as 85° and bacteria at 88° (Brues, 1939). Hindle (1932) showed that certain amoebae found in thermal waters will stand a temperature of 53–54°. He discusses the experiments of Dallinger and Drysdale, in which by a slow increase of the temperature extended over a period of several years the thermal resistance of certain Protozoa was increased and their descendants were finally able to live at a temperature of 70°. Multicellular animals rarely endure more than 48° in a water medium; their maximum temperature lies at about 51–52°, though in some cases resting stages may tolerate a little more (Brues 1939).

While, therefore, the upper temperature limit that can be withstood by organisms lies tolerably near to their normal temperature, such is not the case for the lower limit, if we consider certain small forms and micro-organisms. Below freezing-point living organisms and their eggs or spores find themselves in dry surroundings, a fact which probably counts in their power of surviving at low temperatures. Staphylococci, which at normal temperatures only live for 5 days, were found to die in an ice box after 100 days, but to be still unchanged in that time at a temperature of −188° C. (liquid air) (Kolle *et al.* 1931). Becquerel (1936) carried out some very interesting experiments to study the capacity of micro-organisms to endure desiccation and cold. Test-tubes containing a little soil were dried in a vacuum over barium hydroxide for 3 months. Some of the tubes were then sealed by melting the open end, while others were closed only by a cotton-wool plug. In the first-mentioned case the air pressure had been lowered to 1/100,000 mm. of mercury. In addition, algae were investigated which had been dried and kept *in vacuo* in tubes sealed 25 years earlier. These tubes were immersed for 7½ hr. in liquid helium and a little later in liquid nitrogen for 20 hr. The organisms had thus been subjected to temperatures of −269° C. and −196° C. respectively. With all possible precautions the contents were taken out of the test-tubes and cultured for some time on a sterilized medium. These algae which had been resting for 25 years, began to grow anew, and from the earth in the other tubes there developed algae, Flagellata, Amoebozoa, Heliozoa, Infusoria, Rotatoria, Tardigrada and Nematoda. These forms had consequently been subjected to drought, very intense cold, and in some cases very low barometric pressure, but in spite of these most unfavourable conditions they had, in anabiotic stages or as spores, been able to survive and to continue their life when the environment again became suitable.

If the distributional barriers encountered by small organisms in the air in the form of drought, cold and a low barometric pressure are not necessarily catastrophic for them, this is not equally true of other environmental factors. Many investigations have shown that the sun's radiation has the power of rapidly killing bacteria and various other micro-organisms. The ultra-violet rays of the spectrum, extending between 400 and 290 mμ are those most biologically active. Most important of these are the so-called Dorno rays (shorter than 320 mμ). According to Coblenz & Fulton (1925) ultra-violet rays seem to be most bactericidal at a wave-length between 280 and 170 mμ. This region of the spectrum is gradually completed at altitudes from 20 to 80 km. The region from 280 to 230 mμ appears soon after 20 km.

Diffuse radiation has less influence on micro-organisms than direct sunlight. According to one investigation tubercle bacilli lived for more than 16 days in diffuse light, but were killed in 6 days by direct sunlight. Placed at a distance of 30 cm. from a 30 amp. ultra-violet lamp tubercle bacilli were killed in 3–6 min. Other investigations indicate still greater sensitivity to sunlight. Bacteria of sputum dried on sand were killed in 10–70 days in diffuse daylight, but in 10 min. to 7 hr. in sunshine; in a humid medium they lived for 6 months. Sensitivity to ultra-violet rays has also been shown for Rhizopoda and Ciliata (Kolle, *et al.* 1931).

The intensity of ultra-violet radiation is very different at different positions of the sun. It is greatest in the middle of a summer day, and decreases rapidly in the morning and afternoon, and also in winter. Götz (1926) found an eight times lower intensity of radiation at midday on 15 December, as compared with the same time on 15 June. In cloudy weather ultra-violet radiation decreases considerably (Lunelund, 1945). In high mountains its intensity increases. Thus at Arosa (1870 m.

above sea-level) it is about twice as powerful as at Schömberg (620 m.) (Jüngling, 1938, Fig. 20). The spectrum at very high altitudes of over 20 km. extends farther towards short wave-lengths and the bactericidal power is correspondingly increased. Cosmic radiation will also play a considerably greater part in higher and more rarified air strata.

Here the question arises as to whether all organisms are influenced unfavourably at higher altitudes by the radiation which they encounter. For forms such as small insects and spiders this is probably not the case. Their air transport is, as shown above, generally carried out at moderate altitudes, under 4000 m., and at these levels the animals were often captured alive. Nevertheless, it might be thought that even if they can endure the radiation themselves their reproductive organs may be injured, thus preventing them from having normal offspring. This, however, is not probable as many small forms have a very wide distribution, which is evidently a result of wind transport. Moreover, a comparatively rich fauna has been found on mountains at high altitudes. Thus Sjöstedt (1910) collected no less than 7000 specimens on the African tropical mountains at altitudes between 1600 and 4000 m., including beetles, flies, grasshoppers, earwigs, Collembola, opilionids, spiders, thrombidiids and other mites, pseudoscorpions and land isopods. On the leaves of the sparse plants small snails (*Vitrina*) with transparent shells were creeping about. However, this belt is as a rule enveloped in a foggy mantle, a circumstance which must considerably decrease the intensity of radiation, and mitigate the violent temperature variations and the evaporation which in this thin air become dangerously strong. On the Himalaya the normal occurrence of plants, in foggy areas, stops abruptly at about 5500 m. (Ruttledge, 1934, p. 308). Animals rarely live higher than 5200 m. Altitudes above 6000 m. are only exceptionally inhabited or visited by living organisms. One Himalayan plant has been found as high as 6400 m. (Smythe, 1932, p. 357). Flying condors may reach heights of 7000 m. and the highest camp of the 1924 Everest expedition, 8200 m., was visited by foraging birds (Hingston 1925). Butterflies and spiders found at very high altitudes (6700 m.) have certainly been blown thither.

When one approaches the dimensions of micro-organisms, however, the sensitivity to radiation seems to change.* As shown above, direct sunlight kills micro-organisms, and especially pathogenic ones, much faster than diffuse daylight. Increased humidity affords a certain amount of protection against sunlight. Consequently micro-organisms floating in fog or in a cloud live longer than those subjected to direct sunlight. At high altitudes, owing to frost and the absence of clouds, floating organisms are subject to considerable desiccation. This desiccation is in itself not directly dangerous, but it makes the micro-organisms more sensitive to the influence of radiation. This has been shown by experiments and is probably due to the sun's rays not being absorbed so much in a dry medium (Wiesner, 1907).

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* Spore-forming micro-organisms in particular may under favourable conditions sustain transport at moderate heights, as Proctor (1934) succeeded in growing about 50 % of moulds and bacteria collected over 2700 m. and 35 % of those collected over 4500 m. All these species, however, were non-pathogenic.

As micro-organisms are especially sensitive to the ultra-violet part of the spectrum, since these rays increase in power at higher altitudes, and since the spectrum in very high layers extends farther towards the short waves which are strongly bactericidal, the sun's rays at high levels become increasingly destructive to micro-organisms. Consequently we can state that while the lower cloudy air strata—let us say under 3000–4000 m.—form a suitable medium for the transport of micro-organisms, the higher layers are very inhospitable to them, not so much because of the low temperature, drought and barometric pressure as because of destructive radiation.

Cosmic radiation hardly plays any part in this connexion. The rather unimportant biological effects which experiments indicate are confined to certain questionable influences on retarded development, accelerated growth of tumours and increased frequency of mutations. It is true, however, that there is a large increase in cosmic radiation at increasing altitudes; it is twelve times more intense at 5000 m. than at the earth's surface, and sixty-three times stronger at 10,000 m. (Eugster & Hess, 1940). Nevertheless, the increase at levels where smaller forms can be transported alive is inconsiderable and, especially when it is a question of transport for short distances at moderate altitudes, should not play any measurable part.

V. DISCUSSION

We may state that large organisms, between a length of a few millimetres and about a decimetre, are rarely transported by the air. When air transport is reported for these forms it is always by means of violent winds or the heavy rains that follow them. Consequently, we can only presuppose a regular wind distribution for them in those rather limited areas where such winds are wont to occur. Nevertheless, one must remember that the cyclonic areas were different in the Glacial Period and that at that epoch an aerial distribution was possible along courses which are now out of the question.

Small forms, that is, organisms varying between a length of a few millimetres to about $\frac{1}{10}$ mm., seem to have great possibilities for distribution through the air by convection winds due to differential heating of the air. Insects and spiders, thanks to their chitinous armour, can, under suitable environmental conditions, survive unharmed in the air for one or several days, and might then again reach terra firma alive (e.g. the aphids in North-East Land). Others cannot survive transport through the air-sea themselves, but have resistant eggs which endure protracted desiccation and which often, besides, are provided with hooks, threads or sticky surfaces, fixing them to leaves or grass blades blown up into the air. In some cases they may be transported attached to flying birds. Some large forms may also be distributed by birds. As a rule it is eggs which are thus carried but even fully grown larger forms such as leeches may use this mode of distribution.

Still other forms, on the border line of micro-size, may have an anabiotic stage during which they are very resistant and can pass unharmed through the air-sea. It is significant that many of these animals are hermaphrodite or parthenogenetic. They have thus freed themselves of the necessity of two sexes and they can therefore produce offspring from a single individual when they come down into suitable surroundings.

Microscopic organisms have a high resistance to unfavourable factors met with during transport through the air-sea. In supporting low temperature, drought and low barometric pressure they are superior to all other forms, and, if the factors mentioned were the only impediments they ought to be able to support transport between different parts of the universe. The dimensions of their reproductive bodies may be so insignificant that they would be driven to other planets or even to stars by radiation pressure. Nevertheless, here time comes in as an important factor in the possibility of transport. In spite of the enormous velocity of a particle impelled through space by radiation pressure it would take 9000 years for it to reach the nearest star. To our nearest planet the time should be only 20 days (Petterson, 1944). A presupposition in such a case would be a starting velocity of several thousand metres per second, a circumstance which seems highly improbable. One must also take into consideration the very unfavourable conditions presented by the ionized strata of the atmosphere (Lysgaard, 1943) where probably all living beings are exterminated.*

Even at the earth's surface micro-organisms are very sensitive to sunlight and especially to ultra-violet radiation. These factors are still more important in the upper layers of the air. Not much is known about the sensitivity of micro-organisms to radiation, but it is evident that while small animals are but little influenced, micro-organisms are much more affected. The eggs of *Artemia* can lie on the edges of desert salt pools in burning sunshine for weeks, months, or even years, without losing their power of hatching, while a micro-organism, or its spores, would long have succumbed. In resting stages such as eggs or other reproductive bodies there are thick protective shells which effectively prevent the entrance of most of the deleterious rays. There is here, moreover, another circumstance which may play a part when micro-organisms are concerned. Since with diminishing size the surface of a body decreases less rapidly than the volume, it follows that with a smaller volume factors acting on the surface become of greater importance. The effect of radiation and especially of the chemically important ultra-violet rays, will consequently be greater in micro-organisms. Moreover, the possibility increases of the injurious rays reaching the nucleus thus affecting the cell's vitality. In bacteria there is no real nucleus and damage caused by radiation will probably occur immediately inside the cuticle which may be of a thickness of fractions of a micron. In tardigrades and rotifers the distance to the epithelial nuclei is at least 1 μ, while in nematodes with cuticle, subcuticle and deep-lying epithelial nuclei it is much more. Moreover, epithelial cells in multicellular animals are subjected chiefly to one-sided radiation. It is possible that this plays a part in the decreased sensitiveness of organisms to radiation with increasing size. At any rate, the fact is that micro-organisms, in spite of their great resistance to other unfavourable factors met with in the air-sea, soon succumb there on account of radiation. This occurs more rapidly in a dry than in a humid medium, the latter seeming to afford a certain protection against the injurious influence of the rays.

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* The nitrogen molecules in the F-layer (altitude 180–500 km.) are ionized to N atoms and the oxygen molecules in the E-layer (altitude 90–130 km.) to O atoms by absorbing the very hard ultra-violet rays of a wave-length of over 100 mμ and about 145 mμ respectively. Hereby enough heat is produced to exterminate all living organisms (Lysgaard, 1943, pp. 23–25). In this process the ozone, occurring at an altitude of about 25–60 km., and absorbing ultra-violet rays of a wave-length between 145 and 290 mμ will also contribute (ibid. p. 25). The ultra-violet rays may in addition ionize the organic compounds of the organisms. The question as to whether living organisms, driven forwards through the universe by radiation pressure, can slip in at night on the dark side of the globe is somewhat beyond the scope of this article. Disregarding the role that may be played by the velocity of recombination by night of the N and O atoms (a problem which is as yet not completely solved, according to information given to me by Prof. O. Rydbeck), it seems, however, to be worth while stressing another view-point. In darkness the driving power of radiation is practically lacking and organisms, possibly from another planet, that may have reached the outermost part of the atmosphere sink very little towards the earth's surface at night. When light returns and their velocity towards the earth's surface is speeded up, they are subjected again to the dangerous influence of the ultra-violet radiation and its ionizing effect.

Large quantities of micro-organisms are driven up into the air to return to the ground with downward winds by night, or with showers of rain. The following example shows that large numbers may be involved. Ehrenberg (1849, pp. 286, 291) at Lyons collected dust deposited by the sirocco wind and found 120 species of micro-organisms. They amounted to one-eighth of the whole volume of the dust. The quantity of sirocco dust left by such so-called 'blood rains' has been calculated at between 5½ and 9 tons per square mile. Consequently, almost 1 ton of micro-organisms per square mile may be precipitated on such occasions (Ehrenberg, 1849, p. 310; McAttee, 1917, p. 218).

As small forms and micro-organisms are constantly deposited on the earth's surface one may well ask why they do not flourish everywhere. The answer seems to be that these organisms are often confined to very special conditions of life, or biotopes, and that the smaller the organism, the smaller is also the volume of the biotope. When the small organisms, or their eggs or spores, reach the ground, they will, therefore, in the majority of cases come down in unsuitable surroundings where they must perish. When they happen to meet with suitable conditions it is still not always certain that they will be able to establish themselves there. During those minutes or hours which pass before they have reacquired full vitality, or have reached a reproductive stage, they are menaced by many perils, and it is only after they have surmounted these dangers that the newly started progeny can be considered to have reasonably secured its existence in the biotope.

The number of invaders certainly plays a part here. In the same way that the human body has the power of overcoming a slight invasion of bacteria, nature too, by predatory animals and plankton-catchers which occur in the biotopes, has means by which the balance is maintained within the biotope and by which fresh intruders are as a rule exterminated, if they only appear in small numbers.

Some forms are more exigent than others on a definite type of environment.*

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* That some larger forms seem to have lost the capacity of being widely distributed is, as pointed out by Hultén (1937), probably due to the fact that during their past history they have become more

When, however, the requirements of a certain micro-organism are fulfilled, this form will turn up sooner or later, an observation often made by those occupied with such groups (cf. Lucks, 1912). The consequence of this will be that for micro-organisms there are no geographical, but sometimes climatic and almost always edaphic barriers and boundaries, i.e. barriers and boundaries pertaining to the water or substratum in which the organisms live (Gislén, 1940).

These forms are, some of them, cosmopolitan or in other cases distributed around the whole globe within a certain climatic belt. Most of the small forms, and this applies still more to the micro-organisms, are very rigidly restricted to a certain environment. But because of their ease of distribution they occur everywhere, at least within a certain climatic belt, when their needs of definite edaphic factors are fulfilled. This is only so, however, provided that there has been sufficient time for the germs, transported through the air, to get a chance of reaching the suitable locality and of settling there. In other words, small forms and micro-organisms are rarely of biogeographical significance; rather they are representative of a certain type of biotope. Micro-organisms, with the same possibilities of distribution through the air as small forms, are more at the mercy of radiation. Pathogenic bacteria, with their very special demands on environments, are influenced and limited in their distribution by the radiation from the sun. This obstacle is especially active during clear days, while, as shown by the experiments referred to above, these forms will hold out longer in hazy or foggy weather.

Pathogenic micro-organisms and their spores float everywhere in the air. They are hindered in their passage between their different biotope areas by radiation, but even if they land alive on a suitable substratum their vitality, the quantity of infection, and the condition of the host plays a part, for specimens of bacteria of the most dangerous diseases are always found on the mucous membranes even of healthy individual human beings. Under favourable external conditions, especially when ultra-violet radiation is diminished, it is very probable that pathogenic bacteria can also pass through the air from one host to another—perhaps over longer distances than hitherto supposed. In any case it is remarkable that epidemics of the respiratory organs, after raging in foggy, cloudy and stormy seasons, disappear after a period of steady clear weather sets in.

VI. SUMMARY

1. Among animals which can be transported by air currents three main types have been distinguished: (*a*) large forms, from a length of roughly one decimetre down to a few millimetres; (*b*) small forms varying between some millimetres and about $\frac{1}{10}$ mm.; (*c*) animals of a microsize, smaller than $\frac{1}{10}$ mm.

2. Large organisms are usually distributed by wind in typhoon and hurricane areas alone. During the Glacial Period, however, cyclonic disturbances proceeded along other routes, in this way creating distributional paths which are now no longer in use.

and more specialized as regards their environment. During climatic changes they have lost those components of their stock that had the power of surviving in different types of climate. During a warm period the cold-loving forms have been exterminated; in a cold period the warm-loving ones. In this way the species is restricted to a more limited area within which its members can exist.

3. Small organisms have considerable possibilities of distribution by convection air currents and winds at moderate altitudes. Examples are given of such distribution over great distances. But as the animals are often strictly specialized ecologically (herbivores, parasites, etc.) they have particular difficulties to overcome in their new surroundings.

4. Numbers of micro-organisms are constantly being driven up into the air to return again to earth in rain showers or downward air currents.

5. Micro-organisms are very resistant to unfavourable factors met with in the air-sea. Some may be distributed through the air in an anabiotic stage. Being often hermaphrodite or parthenogenetic, many of them can give rise to progeny from a single individual which happens to arrive in suitable surroundings. Their resistance to low temperature, low barometric pressure and drought is superior to that of all other organisms. Nevertheless, in comparison to larger forms, they are very sensitive to radiations, especially ultra-violet, which seem to check their distribution more than that of larger forms.

6. The explanation of the fact that the microforms do not flourish everywhere is found in their very strict biotopical specialization. But wherever a biotope suitable for a certain micro-organism exists, that organism will appear there as soon as sufficient time has elapsed to allow it to be transported through the air and to settle in the locality. The numbers which arrive are a factor in the survival and establishment of the invading species.

7. No geographical borders or barriers exist for microforms. They are often cosmopolitan, or else regionally distributed around the whole globe in certain climatic belts.

8. Under favourable conditions, especially in humid air, the harmful influence of radiation is diminished, and microforms may be transported alive by winds over greater distances than in clear and dry weather.

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MYCORRHIZA AND SOIL ECOLOGY

By J. L. HARLEY
Browne Research Fellow, the Queen's College, Oxford

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I. INTRODUCTION

The study of mycorrhizas during the last 50 years has resulted in the publication of a very large number of papers. In most of these an intolerable amount of theory is mixed with experimental results and much controversy has resulted. Ramsbottom (1935) appealed for a more objective approach, but the technique of experimentation has proved so difficult that there has not been an advance commensurate with the hard work done by the many investigators. No good purpose is to be served by over-simplification of the problems, nor by ignoring the ecological background of mycorrhizas under natural conditions. Some of the most valuable papers on the subject have dealt ecologically with the problem, and, especially, with those ecological features which affect the phanerogamic partner in mycorrhizal associations. In this article an attempt is made to approach the problems of ectotrophic mycorrhizas from a consideration of soil ecology. It has been impossible to consider all the relevant works within the space available, and, in those parts of the subject which have been well reviewed, much has been omitted. As far as possible comment on recent theories based upon the experimental work has been relegated to the concluding paragraphs.

One of the most difficult sections of research in mycotrophy is to be found in nomenclature. Even the word mycorrhiza itself is not susceptible of clear definition, and later terms, such as pseudomycorrhiza, are even less satisfactory. Part of the aim of the initial sections of this review is to present a sample of the knowledge concerning the association of roots and micro-organisms in such a way as to enable a clearer impression of the terms mycorrhiza and pseudomycorrhiza to be achieved. By pointing out some of the difficulties of classifying this type of phenomenon it is hoped that some of the controversies may be reduced to simpler terms. No attempt has been made to include the extensive material available on endotrophic mycorrhiza, and the reasons for this omission, apart from lack of space, will, it is hoped, become apparent in the course of the article.

II. THE SPATIAL DISTRIBUTION OF MICRO-ORGANISMS IN SOIL

There exists in the soil a flora which is fairly cosmopolitan but whose components vary, in numerical frequency, with zonal or local variations in soil type and properties. It is important, here, to study the variations, for no soil in a natural state nor under cultivation can be considered uniform. Even in a well-mixed experimental sample, bearing no crop, the surface is in contact with the atmosphere and there is a definite change of aeration with depth. Such changes with depth are expressed, in a natural soil, in horizon differentiation, whether visible horizons are present or not. Again, the incorporation of relatively gross fragments of plant debris does not occur, even in cultivation, uniformly with respect either to depth or to surface area. Further, the presence of roots and of larger elements in the flora and fauna provide local variants, the properties of which are frequently left unconsidered in the observation and analysis of large samples. In studying the ecology of the soil all these variations are important, since they may provide special substrates for particular organisms or reactions. An early classification of the soil fungi by Waksman (1917) grouped them into two types: 'soil inhabitants' which were cosmopolitan soil forms and 'soil invaders' which included those growing upon special substrates and having special physiological demands and which were of more complex distribution. Garrett (1946 and elsewhere) has applied this classification to plant parasites in the soil, and he placed unspecialized and facultative types in the first group and the obligate parasites in the second. Burges (1939*b*), however, writing on soil fungi in general, made four classes: true soil fungi; facultative parasites and primary saprophytes; casual parasites and mycorrhiza fungi; and true parasites. Such attempts at classification are at best only broad generalizations, as may be seen from the experimental results of Thom & Morrow (1937). These observers, who divided soil fungi into two classes—primary decomposers of organic matter and those acting upon residual products of decomposition—pointed out that some fungi belong to both groups. In whatever way we classify the members of the soil flora on the basis of their ecology and physiology it is essential to keep clearly in mind that the structure and activity of a natural or a cultivated soil is the resultant of the interrelation of all types. Terms like 'soil invader' may prove misleading, although useful in some contexts.

These points may be illustrated by examples derived from the study of soils showing clear horizon differentiation. Gray & McMaster (1933), Gray & Taylor (1935) and Gray (1935) have studied the distribution of organisms in podsols in Canada and have shown that the organic horizons are biologically more active than the leached horizons, as measured by the production of carbon dioxide, the rate of nitrification and by the production of ammonia from urea. The greater activity was correlated with increased numbers of bacteria, actinomycetes and fungi in the organic matter horizons. Timonin (1935) also studied virgin podsol soil and showed that total numbers of micro-organisms were greatest in the A horizon and lowest in the C horizon. He further demonstrated that, in spite of the decrease of total numbers, there was a relative increase of potentially anaerobic fungi and bacteria with depth. The biological soil horizons generally conformed with the morphological horizons.

Newman & Norman (1941, 1943) have also shown a clear difference in activity between surface and subsurface populations. The ability of the surface population to produce carbon dioxide from cornstalks mixed into the soil was shown to be much greater than that of the subsurface population. Not only was the peak rate of carbon dioxide production in the subsoil smaller, but also the attainment of this rate was more gradual. On inoculating sterile sand mixed with cornstalks with a small quantity of soil from each depth, the same features of carbon dioxide production were observed. Their results point not only to a decreased size but also to a decreased versatility of the micro-organism population in the subsurface soil. Many such observations demonstrate that the grosser forms of differentiation in soil structure and composition are paralleled by changes in the microflora. The distribution of roots and of micro-organisms is correlated, to an extent varying according to plant species and soil type, with horizon differentiation. Hence a direct effect of roots upon the soil population may be partially responsible for the pattern of distribution of micro-organisms.

III. THE INTERRELATIONSHIP OF ROOTS AND MICRO-ORGANISMS

(1) *General description of rhizosphere effects*

For present purposes the work upon the effect of roots and root activity on the distribution of certain components in the soil microflora is of primary interest. It has long been known that the numerical strength of bacteria, and to a lesser extent of fungi, is greater in the soil surrounding the roots of higher plants than in the soil in general. In the last 20 years this has been frequently confirmed and knowledge of the microflora of the root zone has been much extended. Starkey (1929*a*, *b*, *c*, 1931, 1938) has reviewed much of the relevant work. There is not necessarily a sharp change of population between the soil and the root surface, but the soil, the soil adjacent to the roots and the root surface all contain populations differing in numbers and activity. Usually the activity of the root surface population is greatest and there is a decrease in activity going outwards from this zone. For present purposes we will refer to populations of the three zones studied as the root surface, the rhizosphere and the soil populations respectively. The first two, when grouped together, will be called the root region population.

The effect of roots upon various groups of organisms is not uniform. Bacteria, as a group, very greatly increase in numbers in the rhizosphere and on the root surface (Starkey, 1929–38; Thom & Humfield, 1932; Lochhead, 1940; Lochhead

& Chase, 1943; Lochhead & Thexton, 1947; West & Lochhead, 1940*a*, *b*; Timonin, 1940*a*, *b*). In general, fungi and actinomycetes, although greater in numbers in the same zones, seem to be less affected numerically by the roots (Starkey, 1926*a*, *b*, *c*; Kurbis, 1937; Timonin, 1940*a*, *b*). Numerical changes in these groups of organisms do not so accurately express changes of activity as numerical changes in bacteria do. It is probable that the activity of actinomycetes and fungi is often modified as much as that of bacteria.

These changes in composition of the population near live roots are frequently correlated with changes in activity and numbers of organisms bringing about certain important soil reactions. The following reactions have been reported to be increased in rate in the root region: nitrogen fixation, cellulose decomposition, solution of phosphorus compounds, nitrification, and the release of carbon dioxide (Starkey, 1929*a*, *b*, *c*, 1931, 1938; Thom & Humfield, 1932; Krassilnikov, Kriss & Litvinov, 1936*a*, *b*; Katznelson, 1946). The pH of the root region, too, appears to be maintained at a relatively constant value (Thom & Humfield, 1932; Jahn, 1934, 1935; Kurbis, 1937). It would be quite wrong, however, to assume that roots only stimulate the growth of micro-organisms beneficial to them. Starkey (1931) stressed this point, and a slight consideration of the distribution of soil-borne disease corroborates it. Gerretsen (1937) demonstrated, too, a complicated relationship between host and rhizosphere micro-organisms for manganese. Grey-speck disease of oats was much aggravated owing to the activity of the bacterial population of the roots in soils of low manganese content.

Knowledge of the qualitative changes of bacterial population in the root region has been considerably advanced by Lochhead *et al.* 1938, 1940, 1943, 1947; Lochhead, 1940; Taylor & Lochhead, 1938; Katznelson & Chase, 1944. These observers have concentrated in the main on the rhizosphere and have elaborated a means of classifying the bacteria into several physiological groups. The bacteria are first isolated on soil extract media and then grown on a series of other media such as the following: (*a*) basal medium of glucose and salts, (*b*) basal medium with the addition of amino-acids, (*c*) basal medium with the addition of cysteine, thiamin and biotin, (*d*) basal medium and yeast extract. By this means the bacteria may be classified into types according to a combination of morphological characters and physiological demands for amino-acids or other substances. Using the purely morphological grouping first, it was shown that the percentage incidence of morphological types in soil was relatively little effected by manurial treatments which resulted in great changes of crop productivity (Taylor & Lochhead, 1938). On the other hand, a study of the rhizosphere of different plants showed in every case a changed incidence of various morphological types. Especially noteworthy was the increase of motile and chromogenic forms in the rhizosphere. Here, too, gelatine liquefiers and types affecting glucose increased (Lochhead, 1940). Later work, using a physiological classification, indicated that the roots of flax and tobacco favoured the local increase of bacteria possessing complex requirements especially for certain amino-acids and also for thiamin and biotin. This effect was greatest when the roots were growing in poor soil. Soil rich in organic manure contained a higher percentage of these 'demanding' types of bacteria throughout, and consequently the 'rhizosphere effect' was less (West & Lochhead, 1940*a*).

Other observers (Starkey, 1929*b*; Gräf, 1931; Katznelson, 1946) have recorded a maximum rhizosphere effect at the period of maximum vegetative growth of various crop plants. In some instances there was also a great increase of bacteria in the rhizosphere at the end of the season. The second maximum is due, according to Krassilnikov *et al.* (1936*b*), to the increase of spore-forming bacteria, and Starkey (1938) has commented on the 'explosive' increase of root flora on dead roots and dead root tissues. Harper (unpublished) has indicated that there is a predominance of thiamin-demanding bacteria in the rhizosphere of old *Juncus* roots in peat soil, but not in that of young roots. The effect of root species and root condition seems usually to predominate over the influence of soil variation within wide limits. Timonin (1940*a*) has shown that the rhizosphere of oats is relatively richer in fungi than that of alfalfa, which contains bigger numbers of bacteria; while Kurbis (1937) has stated that the differences in rhizosphere population of one species of plant grown in a range of soils is less than the difference between the populations of rhizospheres of different species. In this context the work of Eggleton (1938) is of importance since he estimated the fluctuations of the micro-population of soils weekly over a considerable period. He concluded that long-term changes in numbers are closely associated with the amount of growth and activity of surface vegetation and he thought that similar factors working in conjunction with a direct effect of season on micro-organisms might partially explain the shorter season fluctuations.

More detailed studies of the effect of roots of different genetic origin have been made in connexion with the study of resistant varieties. Thom & Humfield (1932) noted the vast increase of micro-organisms near the roots of susceptible tobacco plants on soils infected with root-rot. Much smaller increases occurred in the rhizosphere of unsusceptible roots. Similar observations have been made by others as Lochhead (1940), Lochhead *et al.* (1940) and Timonin (1940*b*, 1941). In spite of clear-cut specificity of roots in their effect on local soil population, there are, however, variations within the root system of a single plant. Reference has already been made to the difference in the micro-organism population of young and old roots. A similar difference is to be expected between the apical and proximal parts of the same root; and this has been confirmed by Harper (1947 unpublished) to be true of *Juncus* roots. Simmonds & Ledingham (1937) studied the variation of the fungus flora of the roots of two varieties of wheat with depth. They isolated members of twenty-seven genera from the root system and the sub-crown internode. The numbers isolated for roots in the top foot of soil including the sub-crown internode of both varieties, greatly exceeded those in the equivalent sample below. Of the total fungi isolated, 50% were potential wheat pathogens, the vast majority of which occurred in the top foot of soil only. Parasitic lesions, when they occurred, were restricted to this zone too. Hence the differential distribution of micro-organisms within one root system results from both differences of physiology and differences of environment of the roots. It is possible that aeration and carbon dioxide concentration, which exert a direct effect upon root activity and upon fungi, are partly responsible for these variations. A combination of such factors together with variations in the distribution of humic substances could be suggested to explain the recorded variations in the frequency of organisms in podsol soils.

The great concentration of potential disease-fungi in the rhizosphere which was observed by Simmonds & Ledingham (1937) is by no means of rare occurrence, at least in cultivated soils. It is especially prevalent in 'sick soils'. For instance, Berkely & Lauder-Thompson (1934) found that five out of fourteen fungi from strawberry roots in root-rot soils were potentially pathogenic. Similar results have been observed by others (Truscott, 1934; Hildebrand, 1934; Koch, 1935; Hildebrand & West, 1941) and seem to confirm that the survival and spread of pathogens in the soil is aided by the presence of potential host roots and that such fungi may exist in the rhizosphere and on the surface of healthy roots without causing disease. The special subject of root-disease fungi and their survival has been summarized by Garrett (1934, 1938, 1944, 1946).

Kurbis (1937) examined the fungi in the root region of the ash tree. He obtained samples of roots from many localities and convinced himself that a state which might be described as mycorrhiza did not exist. Nevertheless, there were consistently present satellite fungi which, although frequent on the root surface, were relatively rare or absent from the soil. Two in particular *Cylindrocarpon radicicola* and *Fungus imperfectus* I (probably *Rhizoctonia sylvestris*) were always present in the rhizosphere, although he found them absent from the soil; others were not so clearly defined in their distribution. An examination of the root region of beech yields similar data. The mycorrhizas are colonized on their sheath surface by many fungi amongst which are certain very frequent species which, although relatively rare in other soil habitats, are consistently present on the roots in a number of localities.

(2) *Possible explanations of the rhizosphere effect*

In looking for an explanation of the rhizosphere effect of roots on the soil micropopulation, Garrard & Lochhead (1938) suggested the following possibilities: (*a*) food is provided by dead root cells, (*b*) roots excrete decomposable products, (*c*) roots excrete growth-promoting substances, (*d*) roots release carbon dioxide, (*e*) the pH of the root is relatively constant.

Each of these activities or conditions are known to exist. The effect of the sloughing-off of dead tissues from roots was suggested by Starkey (1938) after direct observation of the rhizosphere under the microscope, as a possible factor in the increase of some micro-organisms. The increases of certain types of bacteria, especially cellulose-decomposing and thiamin-demanding types, on old roots has already been noticed and might be connected with the presence of senescent cells. The release of carbon dioxide by roots, especially during active absorption, is well known, and it has been suggested that the dissolution of phosphates and other inorganic substances in the soil is dependent in some degree on carbon dioxide production. The pH of the root zone is constant within narrower limits than that of the soil for any given species (Kurbis, 1937; Jahn, 1934, 1935; Thom & Humfield, 1932).

Interest has recently been aroused to a greater degree in the subject of root-excretion,* for here some explanation of the relatively specific action of certain roots may be found. Work on root excretions of a toxic and non-toxic nature has been reviewed by Loehwing (1937), but little of the information is easily related to rhizosphere effects. It is sufficient, however, to confirm that on the root surface and in the soil immediately surrounding active roots a series of environments for micro-organisms exists which undoubtedly differ markedly from those available in the rest of the soil.

In this context one recalls at once the demonstration of Melin (1925), following Hansteen & Cranner, of the effect of exudates from the tissues of coniferous plants on the growth of mycorrhiza fungi. Whether the identification of the active principle of these exudates as phosphatides was correct or not is uncertain; nevertheless, their stimulating activity is undoubted. Recently Lundegardh & Stenlid (1944) have identified the exudation products of pea and wheat roots grown in liquid culture and have suggested that the presence in such exudates of flavanones and nucleotides may be important in the study of root-inhabiting micro-organisms. It is of interest, too, that of the two types of plants studied, the wheat alone produced flavanone, while the pea produced none; and that the pea produced less sugar and more nucleotides than wheat under the conditions of the experiments. These results were demonstrated both by spectroscopic and microchemical analyses. It is attractive at this point to refer back to the work of Timonin (1940*a*, *b*) who showed that a legume (alfalfa) had a richer bacterial flora on its roots than a cereal (oats), which had larger numbers of fungi. The work of Lochhead and his associates mentioned above clearly indicates, too, that many of the bacterial components of the root population have demands for complex materials such as amino-acids and vitamins.

West (1939) has examined the root excretion of two kinds of flax, namely 'Bison' unsusceptible and 'Novelty' susceptible to *Fusarium* wilt. The plants were grown in sterile water-culture and the concentrations of thiamin and biotin in the culture solution containing the exudates were examined after periods of 1 and 2 weeks. In both cases definite amounts of both substances were identified, but there was no significant difference between the two varieties in the release of these two vitamins. It is clear that, although the release of these two vitamins may be important in the general differentiation of the rhizosphere populations of flax, their presence in exudations affords no explanation of the differences between resistant and non-resistant varieties. Timonin (1941) also worked on the rhizosphere population of these same two flax varieties. The two varieties supported different rhizosphere populations of fungi and actinomycetes. He further demonstrated that there were differences between the varieties in the frequency of fungi and actinomycetes in the rhizosphere at different soil water-contents. By an ingenious means the root excretions of flax plants grown in sterile culture solutions were collected and fed into celloidin cylinders which were immersed in soil. Around these cylinders, with a very slow current of root extract percolating through them, artificial rhizospheres were set up. After these artificial rhizospheres had been colonized by soil micro-organisms, the constitution of their population was estimated. The differences between the populations of the artificial rhizospheres and the soil were similar to the differences observed between real rhizosphere populations and the soil.

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* The terms 'excretion' or 'exudation' are used for convenience only. Apparent excretory products may be produced by senescent cells, but, in any case, the ecological effect would be similar.

This experiment demonstrated the important effect of root excretion in the determination of the constitution of the rhizosphere population. Further experiments by the same observer demonstrated also that these same root excretions exerted a direct effect on some of the fungi of the rhizosphere in pure culture. For instance, the exudate of Novelty flax, the susceptible variety, stimulated the growth of *Fusarium* and *Helminthosporium*, whereas the exudate of Bison flax did not. However, Bison flax exudate stimulated the growth of *Trichoderma*. Analysis of the excretions showed a significant concentration of hydrocyanic acid in that of Bison flax, and this substance in low concentration has the effect of stimulating *Trichoderma* and depressing *Fusarium* and *Helminthosporium* in pure culture. Timonin's demonstration of the presence and effect of hydrocyanic acid confirms the results of Renolds (1926) who showed that this substance, originating from cynogenetic glucosides, produced by wilt-resistant flax, inhibited or depressed the growth of *Fusarium lini* in pure culture.

In the study of resistance to parasitic attacks on roots further clues may be obtained to the problem of specificity of rhizosphere effects. For example, Ezekiel & Fidge (1938) made an attempt to determine the reason for the non-susceptibility of many monocotyledons to *Phymatotrichum* root rot. They extracted the root tissues of monocotyledons and dicotyledons and demonstrated that the juice of both groups of plants contained substances inhibiting *Phymatotrichum* in pure culture. In dicotyledons, however, only one inhibiting substance, soluble in water, was present; whereas in monocotyledons there occurred in addition an extremely active inhibiting substance in the ether-soluble fraction.

While such results may be of interest in the study of disease, the effects of tissue extracts are also of interest in the study of some of the surface phenomena on roots. They may, for instance, be of importance in a consideration of mycorrhizal infection, especially endotrophic and ectendotrophic forms. For example, Burges (1939*a*) demonstrated that the contents of cortical cells of *Orchis incarnata* were capable of bringing about deformation, inhibition, plasmolysis and digestion of the endophytic fungus growing in pure culture. This property may well be considered as functioning in the prevention of the colonization of the whole cortex by the fungus. The recent discovery of antibiotic substances in dried root material of many higher plants emphasizes their widespread occurrence. If future work shows them to occur in root exudates it will open up new lines of study on rhizosphere effects and disease resistance.

IV. THE PLACE OF MYCORRHIZAL FUNGI IN THE RHIZOSPHERE

(1) *Mycorrhizal phenomena as a special case of rhizosphere effect*

Enough has been written of the general interaction between roots and micro-organisms to suggest that those relationships between roots and fungi to which the name mycorrhiza has been applied must be considered as special cases of a more general series of phenomena. Amongst the mycorrhizal phenomena two main types have been described. The ectotrophic form has a well-developed sheath of fungal pseudoparenchyma enclosing the root, fungal hyphae penetrating between cortical cells but few into them. The endotrophic form, by contrast, has the fungus mainly within the tissues, inhabiting the cells of the cortex, where it may be digested by the protoplasm of the host. It is not surprising from what we know of rhizosphere and root-surface populations that these two forms are by no means clearly delimited one from the other and there are innumerable intermediates. In addition, Peyronel (1922, 1923, 1924) has described conditions arising by double infection, first by a phycomycete and secondly by a rhizoctonia; and many of the so-called cases of endotrophic mycorrhiza are greatly variable in the intensity of infection. Such is the case, for instance, in strawberry roots where a phycomycete and a rhizoctonia have both been recorded, and there may also be present a large number of other fungi, some potentially parasitic, on the root surface and in the rhizosphere (Hildebrand, 1934; Hildebrand & West, 1941). Similar variability was observed by Burges (1936) in the infection of *Schellhammera undulata* in various soils in Australia. The application of the name mycorrhiza to many endotrophic root infections is likely, therefore, to present some difficulty unless there is clear evidence that the association is truly one of mutualism, and we accept this as a criteria for the application of the name. Such evidence would be of consistent intracellular digestion of the fungus or of the possession of physiological properties which would not be expected in the other types of root and fungus interaction, such as parasitic attack or rhizosphere effect.

The ectotrophic mycorrhizas of forest trees, to which the name was first applied, are easier to differentiate from other types of interrelationship between roots and fungi. The relatively constant morphological form of any given species of infected root, the presence of the external fungal mantle and internal Hartig net, the hypertrophy of the cortex and mode of branching, are so clearly characteristic that these structures are, not infrequently, termed synthetic or composite organs. In spite of this, the term pseudomycorrhiza has been coined. It is used to cover a wide variety of ectotrophic and endotrophic associations which depart either in morphological form or in presumed physiological function from what is considered normal for any species of root in association with fungi. In all these ectotrophic forms, whether mycorrhizal or pseudomycorrhizal, the micro-organism population of the root surface and the rhizosphere appears to be dominated by the activity of one or few species of fungus. Examination of the satellite population of mycorrhizal roots has been made by Jahn (1934, 1935) who showed that surrounding the mycorrhizal sheath was a 'peritrophic' population which was somewhat different in acid and basic soil. His rather teleological outlook led him to ascribe to the peritrophic fungi the function of 'living buffers' which tend to keep the root region at constant pH. It seems clear too, from work on beech root fungi, that the population of the root region of mycorrhizal roots of this species can be divided into two parts different in composition: the population of the sheath surface and the population of the rhizosphere. Both these components of the root flora seem to differ quite markedly from the population of the surrounding soil horizon.

We are dealing, then, in ectotrophic mycorrhiza with a special case where, on a given root, one species of fungus in particular is so affected by local conditions as to become dominant in the root-surface zone. We can only conclude, from what we know of the mutual competition and antagonism between species of micro-organisms, that the whole population of the root region is, in consequence, profoundly modified. Associated with this modification of the population there occur the effects on the host roots already briefly described. It would be surprising if such a series of changes were without effect upon the local soil processes and without effect upon the functions of the host plant; but such a view has supporters.

In the great majority of cases that have been carefully examined the sheath-producing organisms of ectotrophic mycorrhizas are basidiomycetes belonging to such genera as *Amanita*, *Boletus*, *Tricholoma*, *Cortinarius*, *Lactarius*, *Russula* and *Paxillus* of the Hymenomycetes and to others of the Gasteromycetes. Two main methods have been used to confirm these fungi as mycorrhiza-formers (Melin, 1936). First, by isolation of the sheath fungi into pure culture, their reinoculation on to the roots of the host plant and their subsequent identification in mycelial form as basidiomycetes. Secondly, by the isolation into pure culture of known basidiomycetes by using inocula derived from tissues of the sporophore and testing them under aseptic conditions in culture with tree seedlings. When such attempts at formation of mycorrhiza in pure culture are successful, the fungi may certainly be regarded as potential mycorrhiza-formers; but negative results under artificial conditions are not necessarily significant. Fries (1942) has recently shown that haploid as well as diploid mycelia of some species are capable of forming typical sheaths and Hartig nets. It is clear, therefore, that some of the imperfect mycelia isolated from ectotrophic mycorrhizas may be haploid basidiomycete mycelia, although not yet confirmed as such.

Although some species of all the genera mentioned above have been confirmed as potential mycorrhiza-formers, there is a degree of specificity towards the host species, and it has been well known for a long time that perfect stages of certain species are restricted to localities where certain trees occur. The experimental work demonstrated further that the ability of any fungus to produce mycorrhizas depends, to some extent, on external conditions. The recent work of Modess (1939, 1941) and Lindquist (1939) especially stresses this point; but a full experimental demonstration of the degree of specificity and of variation in ability to form mycorrhiza, of any one basidiomycete, has not yet been made. In addition to the fungi which form fully developed ectotrophic mycorrhizas, many may occur on the root surface either as dominants (e.g. *Mycelium r. atrovirens* and *Rhizoctonia sylvestris*) or as frequent members of the flora which do not form a complete sheath, or fail to form an intercellular net, or to colonize or parasitize the root cortex. Others capable of forming perfect mycorrhizas have been recorded as parasitic under some external conditions.

A study of the physiology of the basidiomycetes must therefore be fundamental to a study of ectotrophic mycorrhizas, and, owing to the extensive work performed in recent years, especially at Upsala by Melin and his associates, our knowledge of this subject is becoming wider.

(2) *The physiology of the mycorrhizal fungi*

Although the great majority of ectotrophic mycorrhizal fungi are believed to be basidiomycetes, not all the fungi of this group are capable of forming mycorrhizas. There is a considerable diversity of physiology amongst them. The species inhabiting woods and forests may be divided roughly into those causing primary decomposition of litter and other debris, and those associated with the roots. The majority of mycorrhiza-formers examined by Melin (1925) required sugars such as glucose as a source of carbon, and some made fair growth on maltose and xylose, and on the alcohol mannitol. None grew strongly on starch or inulin and no growth occurred on cellulose. These results have been confirmed by later workers (e.g. How, 1940) so that we are very probably dealing with a group of fungi which can colonize effectively only those substrates or soil locations where there exists either a significant free concentration of sugars or where sugars or simple organic compounds are being consistently released. In this respect these particular basidiomycetes differ sharply from those destroying litter and wood, which can bring about the breakdown of lignin and cellulose, or lignin alone, with considerable rapidity (Lindeberg, 1944; Harris, 1945; Lindeberg, 1947).

The nitrogen nutrition of mycorrhiza-formers does not seem to differentiate them as clearly from other basidiomycetes as do their carbon requirements. For almost all ammonium salts of both organic and inorganic acids are suitable nitrogen sources, and for most of these fungi nitrates are inferior as a source of nitrogen under conditions where great change of acidity does not take place. Some appear unable to use nitrates at all. Substituted ammonias, amides, amino-acids, urea, nucleic acids and some proteins are also suitable nitrogen sources for some species (Melin, 1925). Similar nitrogen demands have been demonstrated for soil and wood inhabiting types (Treschow, 1944; Lindeberg, 1944). Melin also demonstrated that sterile extracts of humus obtained by filtration formed an adequate substrate for several mycorrhizal fungi if glucose was added to it. Growth was equivalent in quantity to that on a synthetic medium.

Later Melin (1946) showed that the effect of litter extract might be fourfold, namely: (1) provision of nutrients; (2) provision of ash compounds; (3) provision of organic accessory growth factors; and (4) provision of substances inhibiting or depressing growth. He eliminated the effect of the first by applying extract in minute suboptimal amounts to his cultures and he then determined the ash effect by giving parallel cultures doses of ash. The ash effects varied in magnitude with different species but were probably ascribable, when present, to calcium and magnesium contained in the ash. The former element seems to be essential to the majority of basidiomycetes, at any rate in traces (see, for instance, Treschow, 1944, and Lindeberg, 1944).

Over and above the effects of (1) and (2) there was a further stimulation of growth due probably to some accessory growth substance contained in the litter extract. There was present, too, a substance which depressed the growth of some of the fungi. This inhibitor, or depressor, was soluble in cold water, so that in the presence of large doses of cold-water extract the growth of mycorrhizal fungi was much reduced in rate. Litter-decomposing types were relatively insensitive to the inhibiting substance.

These inhibitors, derived from newly fallen litter, were very probably of a different nature from those which Melin and others have found in heat-sterilized humus. For instance, Melin in his early work had observed that whereas mycorrhizal fungi would grow on partially sterilized humus, no significant growth occurred on humus fully sterilized by heat. If fully heat-sterilized humus was leached with water, growth was again possible. This result was believed to be due to the removal of substances depressing or inactivating growth, which had been produced by heating the humus. The effect, however, may not be a simple one, for, in partially sterilized humus, residual micro-organisms may both remove or neutralize any depressant substance and also provide soluble metabolic products which allow the fungus to grow. This point is further emphasized by the fact that, in partially sterilized humus, growth was much more vigorous than in fully sterilized and leached humus, although the basidiomycete was soon overgrown by competitors.

Considerable interest has centred on the demands of mycorrhizal fungi for accessory food factors, such as the B vitamins. All that have been studied carefully are heterotrophic, completely or partially, for thiamin or for one or both of its components (Melin & Lindeberg, 1939; Melin & Nyman, 1940, 1941; Melin & Norkrans, 1942; Melin, 1946). A few are also stimulated by biotin or inositol in the presence of thiamin. There seems to exist in yeast extract (Melin & Lindeberg, 1939) and in newly fallen litter (Melin, 1946) further growth-promoting substances which are not replaceable by any of the pure substances tested. Such needs for accessory food factors are by no means peculiar to the mycorrhiza-formers. Many other fungi, both litter-decomposing and wood-decaying species of basidiomycetes and of other groups, have similar demands (Fries, 1938; Schopfer & Blumer, 1940; Treschow, 1944; Rennerfeldt, 1944; Lindeberg, 1944, 1946*a*, *b*). Steinberg (1939) and Robbins & Kavanah (1942) have summarized most of the information on the vitamin demands of fungi in general.

Fries (1941, 1943) has pointed out a further effect of accessory factors. Many of the mycorrhiza-forming basidiomycetes are difficult to germinate under experimental conditions. They differ in this respect from the majority of litter- and wood-destroying species. Fries has demonstrated experimentally that successful germination may be obtained by growing colonies of certain yeasts and non-spreading micro-organisms on the agar on which the spores are spread to germinate. With *Torulopsis sanguinea* and other yeasts he successfully germinated and grew a number of monospore cultures of several species of basidiomycetes. The presence of fungi, such as *Cladosporium* and an unnamed species, also encouraged the germination of *Lycoperdon umbrinum*, and the presence of *Mycelium r. atrovirens* and *Cennococcium graniforme* encouraged the germination of some of the boleti. The effects of the associated organisms could not be simulated by using vitamins and other pure chemicals, but could, in the case of some of the boleti, be brought about by fruit-body extracts.

A careful consideration of this information on the physiology of mycorrhizal fungi inevitably leads to the conclusion that it does not yet afford an explanation why these fungi should form mycorrhizas, why they should exhibit a degree of specificity to given host roots, or why they should cause the observed effects on those roots.

None of the interesting properties of these fungi detailed above is exclusively theirs. The demands for simple sugars, vitamins and other growth factors in particular are held in common with many other rhizosphere and root-surface micro-organisms which do not form mycorrhizas. Suggestions that partial vitamin heterotrophy of both fungus and host explains mycorrhiza formation cannot yet be seriously considered, and widely divergent views are put forward. For instance, Lindquist (1939) and others suggest that the fungus derives from its host, amongst other substances, the B vitamins; yet McDougal & Dufrenoy (1940) are of the opinion that B vitamins are derived from the soil by the activity of the fungus and translocated to the root. It is likely that both organisms need these vitamins, but their origin is as yet unknown. Not only have they been shown to be produced by biological activity in the soil, but they are also extractable from decaying plant material under experimental and natural conditions. Vitamins are also present in solutions in which some sterile seedlings have been grown. Moreover, Björkman (1942) has shown that watering soil with thiamin or biotin has no effect on mycorrhiza formation, and this point recalls the less sharp effect of vitamins upon the rhizosphere population than that of other substances (Lochhead & Thexton, 1947). Certain observers have, it is true, demonstrated the effect of root exudates and root extracts on fungi associated with mycorrhizas (Melin, 1925; Harley, 1939*b*; How, 1941), but these will remain of limited value until the substances are demonstrated to have a specific action on the mycorrhizal fungi only, or to have a definite modifying effect on the rhizosphere population as a whole. Kurbis (1937) has demonstrated a stimulating effect of ash-root extract upon *Cylindricarpon radicicola*, which, although constantly present upon the ash roots he examined, did not produce mycorrhiza with these roots. This indicates the need for regarding the results of such work as of limited value at the moment.

Our knowledge of the physiology of the mycorrhizal fungi, although not yet producing any solution to mycorrhizal problems, further stresses the importance of considering ectotrophic mycorrhizas as part of the wider subject of the interaction between micro-organisms and roots, for the fungi concerned have demands for special growth substances similar to those of the important bacteria and fungi of the rhizosphere and root surface. Nor can we yet distinguish clearly any physiological activity of these fungi which may be particularly regarded as important to the host plant. They have not been shown to bring about peculiar changes in essential soil nutrients although the micro-organism population of the root region may, as shown above, change the availability of nitrogen, phosphorus and other soil compounds.

V. THE CONDITIONS FOR MYCORRHIZA FORMATION

Some information is available concerning the conditions under which ectotrophic mycorrhizas are formed. The subject has been summarized by Hatch (1937) who has himself performed much experimental work on this puzzling subject. Of first importance is the differential distribution of mycorrhiza in any single-root system. The primary roots of any forest tree which forms ectotrophic mycorrhiza are of two main kinds—long roots capable of indefinite growth and short roots of restricted growth in length. It is the latter which take part in the formation of characteristic mycorrhizas. The long roots may become covered, at some time of the year and under some conditions, with a thin mantle of fungal tissue, and, more rarely, the tip may be over-arched by the mantle and may develop an intercellular Hartig network of hyphae. At times both long and short roots may grow through their mantle and renew unrestricted growth. Usually, however, the long roots do not form typical mycorrhizas although colonized to some degree by the mycorrhizal fungi and by other rhizosphere fungi. Short roots, on the other hand, very frequently form typical mycorrhizal associations and undergo characteristic branching and hypertrophy, the details of which depend upon the species of host, the species and condition of the fungus, and the general conditions in the rooting medium. The less usual states of both long and short roots are frequently called pseudomycorrhizas, but they may only differ in degree of development of one or other of the characteristic features of a typical mycorrhiza, or they may differ widely from that condition.

There are, therefore, two types of primary root which behave differently with respect to the soil and its micro-organisms. The difference in behaviour must depend upon differences of physiology of the roots themselves. The problem of the conditions under which mycorrhizas are formed, is therefore complex. Out of this complex we may detach two separate problems: (*a*) What determines the formation of short and long roots? Does the frequency of occurrence of short roots vary with external conditions? (*b*) Under what conditions do the short roots become modified into mycorrhizas? Is there a variation in form and structure of the association between root and fungus with different soil conditions, with different host vigours and with different fungal vigours?

(1) *The conditions favouring the formation of short roots*

It is well known that the relative growth of shoot and root of most species of plant is very sensitive to changes in light intensity and in available soil nutrients. The reduction of relative root growth in soils of high nutrient content and in low light intensity needs no further stressing (Aldrich-Blake, 1930, 1932; Harley, 1939*a*, Björkman, 1940, etc.). Associated with the reduction in relative root growth there is a reduced branching of the roots at low light intensity (Björkman, 1940), and, at very low light intensity (*c*. 4% daylight), no short roots are produced by conifers (Hatch, 1937). The pot experiments of Mitchell (1939) have described the variations of the root systems of white pine with manurial treatment. The roots were most extensive, produced the greatest number of primary and secondary laterals, short roots and root-hairs in sand cultures containing low concentrations in the working range of nitrogen, potassium, phosphorus and calcium. Below such concentrations starvation symptoms involving both root and shoot occurred, whereas above these concentrations the relative root growth and development was diminished. The results of soil analysis by Wilde (1938) and Wilde & Patzer (1940) indicate that relatively high available concentrations of these elements are present wherever successful natural regeneration occurs; and White (1941) has shown that prairie soils bearing trees contain higher concentrations of available potassium and phosphate than do treeless prairie areas. Wilde (1946) suggested that the maintenance of forest nursery soil demands application of fertilizers in greater quantities than is usual in farming practice. It is not surprising, therefore, that other workers (McComb & Griffiths, 1946; Rayner & Neilson Jones, 1944, etc.) have demonstrated a stimulating effect of phosphorus-containing substances on the growth and development of short roots of conifers on soils of rather low available phosphorus content. The effect of such conditions may not be directly upon the roots themselves, for Tyagny Ryadno (1933) has shown that small doses of phosphate may increase phosphate availability in soil by the stimulation of micro-organisms capable of bringing it into solution. Young (1940*a*) has also concluded that the effect on conifers of phosphate doses to the soil may be of an indirect nature. These suggestions concord with the observations that small additions to soil of phosphate compounds in the form of superphosphate or basic slag often produce very startling increases in the growth of trees not easily explained by the weight of the added phosphate material.

An extremely important point arises, however, from Rayner's work (1944), for she noted that the addition of composts or basic slag stimulates seedlings grown on Wareham soil and that a stimulation of root growth and short-root production precedes mycorrhiza formation. We are undoubtedly justified, therefore, in separating the problem of short-root development from that of mycorrhiza formation. Nevertheless, it is as well to mention a further complication. McComb (1938, 1943) examined the populations of conifer seedlings growing in nursery beds where, in local patches, mycorrhiza had developed spontaneously on groups of seedlings. The comparison of mycorrhizal and non-mycorrhizal seedlings from these beds may, of course, be criticized owing to the possibility of sampling errors. However, here we may take the results at their face value. The mycorrhizal seedlings produced twice as many short roots as non-mycorrhizal seedlings. Of these short roots, half formed mycorrhizas and the other half were described as 'uninfected'. It is possible, therefore, that the conversion of a considerable number of short roots into mycorrhizas might have stimulated further short-root production. A similar result with Douglas fir was obtained by McComb & Griffiths (1946) after direct soil inoculation. Whether this possibility is real or not, it is certainly true that the form of uninfected short roots, or of short roots in which either the mantle or the Hartig net is defective, is usually simple and relatively unbranched. It seems probable, therefore, that there is a stimulation of short-root branching when mycorrhizal fungi become truly dominant.

(2) *Conditions necessary for the formation of mycorrhiza by short roots*

Of course the primary condition for the conversion of short roots into mycorrhizas is the presence of a fungus capable of forming the sheath and Hartig net, and of causing the hypertrophy of the root tissue. In some experimental work such fungi have been assumed to be absent from natural soils where no mycorrhizas were formed (e.g. Rayner & Neilson Jones, 1944; McComb, 1943). Nevertheless, it has sometimes been demonstrated, as in the two cases cited above, that suitable fungi were present in the soil but failed to form the composite organs. From this it is clear that external conditions may occur which so affect either the host root or the fungi, or both, that mycorrhiza formation does not take place. The observed variation in the intensity of mycorrhiza formation in nature (see Hatch, 1937) may, therefore, depend on a combination of the following variables: (*a*) the presence or absence of the appropriate fungi, (*b*) the effect of their environment upon these fungi in such a way as to affect their 'virulence', and (*c*) the effect of external environment on the host roots in such a way as to modify their excretion or their internal physiological make-up, or both.

In addition, there is a variation of response due to age of the host plant. Mycorrhizas are absent from the roots of very young seedlings and usually develop at a particular period of growth, provided that conditions are suitable. For instance, Huberman (1940) gives time-tables for the 'normal' stages of growth of several species of pine seedlings. The following example is based on his descriptions for Slash pine, but other species are similar: 0–20 days, germination; 40–60 days, cotyledons open into rosettes, primary needles appear, lateral roots appear; 60–80 days, primary needles increase in number, mycorrhizas appear.

It seems possible that the internal physiological condition of the seedlings undergoes changes during this period, so that only after the seedling has reached a certain stage are mycorrhizas formed. A similar sequence is also observable in the majority of conifers and of some broad-leaved trees. In the case of the beech, mycorrhiza formation in woodland soils seems to follow the expansion of the first pair of leaves.

One of the necessary conditions is certainly the formation of short roots, and in those circumstances, where short-root formation is much depressed, the degree of formation of mycorrhizas is also depressed; but an exact analysis of the factors cannot be made from the data available. It is clear, however, that there are circumstances, as for instance in soil manured with balanced solutions in concentration in the working ranges for conifers (Hatch, 1937; Mitchell, Finn & Rosendahl, 1937), where little or no mycorrhiza is formed although short roots are produced. Again, where there is only a relatively slight deficiency of one or more nutrients, mycorrhiza formation may be delayed to a period later than usual. Hence we arrive again at the conclusion that different internal physiological factors in the root are probably involved in these two processes. The conditions for the onset of mycorrhiza formation are, therefore, reminiscent of the variation in the rhizosphere population and the change of its activities with changes of vegetative condition of the host plant.

Hatch summarized results derived from the work of many previous observers, together with his own observations, in his paper of 1937. The frequency with which short roots are converted into mycorrhizas depends upon the lack of balance of soil nutrients. Deficit of nitrogen, phosphorus, potassium or calcium, or the lack of balance of any one of these, tends, he concluded, to promote mycorrhiza formation. Most instructive, indeed, was his own observation that the roots of pine plants which project through a pot of soil containing balanced nutrients into an infertile soil do not develop mycorrhiza. This suggests that the internal physiological condition arising out of the presence of adequate and balanced available nutrient supply to most of the roots militates against mycorrhiza formation in the remainder. Hatch's infection experiments with pure cultures of fungi showed also that the adsorption of nutrient material on to colloidal particles in the soil might significantly alter their availability. On substrates with a high colloidal content the degree of mycorrhiza formation, even when a complete culture solution was added, greatly exceeded the mycorrhiza formation in sand cultures of low colloidal content.

Nothing in later work seems to argue against these conclusions of Hatch in respect of phosphorus and nitrogen, provided that the other conditions—presence of necessary fungi, adequate virulence of the fungi, a light intensity greater than 4% daylight—are fulfilled. Björkman (1940, 1942), however, disagreed that potassium and calcium availability play a very significant role in mycorrhiza development, although high pH values in the soil might be unfavourable to mycorrhiza formation. He stressed, in addition, the complicating effect of light intensity. For instance, in his experiments pine produced no mycorrhizas below 6–8% of daylight, and relatively few up to 12%. Mycorrhiza reached maximum development between 23 and 49% of total daylight. Mycorrhizas on spruce plants increased gradually in abundance from 0 to 100% of daylight.

Björkman was particularly struck with the similarity of his curves for mycorrhiza development and those of Stålfelt (1924) for photosynthetic rate plotted against light intensity in various conifers. He believes that the free sugar content of the root tissues is an important physiological factor in the determination of mycorrhiza formation. In experiments with aseptic cultures of pines grown in conjunction with *Rhizopogon roseolus* and *Boletus granulatus*, mycorrhizas were formed even in low light intensity if a supply of glucose was added. Under these conditions high nitrogen or phosphorus concentrations in the substrate depressed mycorrhiza formation. The result was believed to be explicable if the addition of these substrates caused an increase of sugar utilization and a decrease of sugar available to the infecting fungus. This belief was strengthened later by experiments (Björkman, 1944) in which pine plants were ringed with wire for periods of three or six months during the growing season. The reducing power of root extracts was determined by the Hagerdoorn-Jensen method, and the frequency of new mycorrhizas formed on the roots was determined in controls and strangled trees. Both root development and mycorrhiza formation was depressed on the strangled plants, but pseudomycorrhizas were increased. A comparison of reducing power (assumed to be due to sugars) of the root extract with the degree of mycorrhiza formation showed a positive, roughly linear, correlation. But this experiment cannot be accepted as conclusive except in so far as the decrease of root activity brought about a decrease of root development and a decreased mycorrhiza formation.

So far the correlation of the degree or extent of mycorrhiza formation with the physiological condition of the root has been described. It is probable—but not inevitable—that these factors are causally related. Nevertheless, change of root physiology and the nature of root excretions may have an effect on the relative dominance of the mycorrhizal fungus only through the associated micro-organisms. It has not been demonstrated that this is so, but the mutual antagonism and stimulatory reactions of micro-organisms are such that this surmise is permissible. It is impossible here to examine the literature on mutual antagonism in any detail, for the subject has been well reviewed and only a few workers have dealt with known mycorrhiza-forming fungi (Wilkins & Harris, 1944; Wilkins, 1945, 1946*a*, *b*; Wikén, 1947; Wikén & Oblom, 1947). This branch of study is undoubtedly of great importance in the ecology of root populations and will need much attention in the future. A direct effect of the availability of soil nutrients or of other properties of the soil upon the microbial population, especially on the prevalence of certain species, might explain the absence of mycorrhizas in some soils. The absence of mycorrhiza formation, owing to the lack of virulence of such mycorrhiza-formers as are present in Wareham soil, has been ascribed to the presence of soil toxins (Rayner & Neilson Jones, 1944; Brian, Hemming & McGowan, 1945) which, it is thought, may be identical with gliotoxin and produced by certain penicillia prevalent in the soil. Again, the parasitic attacks on roots and mycorrhizas by fungi such as *Mycelium r. atrovirens* and *Rhizoctonia* spp., which occur frequently in some soils, may to some extent depend upon the nature of the surrounding soil and its population. The abnormal attacks of true mycorrhizal fungi on roots which are described by various workers (Rayner & Neilson Jones, 1944) may possibly also be determined by similar factors. Romell (1939) and Latham, Doak & Wright (1939) suggested that one of the important effects of mycorrhiza formation was the prevention of pseudomycorrhiza formation by tree roots. No doubt, they implied, the mycorrhizal fungus prevents the parasitic attack by species harmful to the roots.

Further evidence is available that there is a variation of virulence in respect of mycorrhiza formation depending on the prehistory or the genetic make-up of the strain of fungus. Strain specificity to vitamin demand has been demonstrated for several mycorrhizal fungi in the work of Melin and his associates quoted above. There is also some evidence of variation within species of mycorrhizal fungus, so that strains derived from different host species exhibit different characteristics. Fuller consideration of this aspect of fungal action will only be possible when more is known of the reported causation of genetic change by external cultural conditions arising out of the presence of certain chemical substances and of antagonistic associates.

To sum up, then, it is clear that factors affecting the internal concentration of material in the host, such as light and nutrient supply, may affect the nature of the root population and may determine the presence or absence of mycorrhiza, provided that suitable fungi are present. This effect may be brought about through root conditions and excretions or, in part, by soil conditions directly affecting the vigour of the fungi or the associated micro-organisms.

VI. THE INFLUENCE OF THE MICRO-ORGANISMS OF THE ROOT REGION ON THE ACTIVITIES OF THE HOST PLANT

So far, the effect of roots upon micro-organisms has been described so as to emphasize the similarity of the factors which have been invoked to explain both the general case of rhizosphere effect and the special case of ectotrophic mycorrhiza. The evidence, though good as far as it goes, stops short of any real explanation of the different behaviour of the various micro-organisms with different roots. We cannot yet attempt to explain the property of certain organisms to penetrate the root system as pathogens or mycorrhiza-formers, except in rather vague terms. Some instances have been given where a certain fungus has been shown to act either as a pathogen or as a mycorrhiza-former; and again both pathogens and mycorrhiza-formers may act as unspecialized associates. These groupings cannot therefore be regarded as dividing the root-inhabiting organisms sharply into three classes and we are justified in considering the general case of the influence of the micro-organisms of the root region before passing on to the special case of the effects of the mycorrhizal organisms on their host.

The general stimulation of microbial and fungal activity in the root region appears to be of universal occurrence, and with it may occur changes of rate of certain of the soil reactions which are usually regarded as essential for the satisfactory growth of normal green plants. In particular, reactions affecting soil nitrogen compounds, the availability of phosphorus, iron and manganese have been shown to be influenced by rhizosphere organisms. The quantitative increase or decrease in available material in the rhizosphere is often sufficient to produce a significant effect on the host plants, and such effects cannot be disregarded in assessing the value of experimental results where soil inocula are used to introduce particular organisms, or where there is a variation in experimental treatment between the micro-organism population of the control and of other series. Starkey (1931) has reviewed much of the older work on this subject and here it is sufficient to quote a few recent examples demonstrating the importance of both bacteria and fungi in this regard. Kurbis (1937) showed that the introduction of fungi, normally associated with ash trees but not forming mycorrhiza with them, into sterile sand cultures of ash resulted in a great stimulation of growth. His comparison of plants in sterile or near-sterile conditions with those artificially inoculated showed that growth, and especially root growth and branching, was increased in the presence of the rhizosphere fungi. We have here no real evidence of the nature of the activity of the fungi concerned, beyond their effect on the host plant, although Kurbis has suggested that they may have a considerable influence on the pH of the root region. The differences observed by White (1941) in his comparison of the behaviour of broad-leaved trees (*Fraximus Pennsylvanica* and *Ulmus Americana*) on prairie and forest soils could possibly be of a similar nature to the differences of behaviour seen in Kurbis's experimental cultures. Isakova (1936) and Isakova & Smirnova (1937) have investigated the effect of inoculating sterile cultures of crop plants with bacterial populations derived both from the roots and from the soil. Stimulation of germination and of other growth phases was considerable, and a degree of specificity of the effects of different populations was noted. These workers ascribe their results to hormonal stimuli particularly on account of the increase of rate of germination of seed before a nutrient stimulus might be expected to act.

Gerretsen (1948) investigated the effect of micro-organisms on the absorption of phosphorus by various crop plants. His experimental methods ensured sterility and eliminated the complicating effects of nitrate formation and nitrogen fixation. In all cases insoluble phosphorus compounds were brought into solution more readily in cultures infected with bacteria than in sterile cultures. Local dissolution of phosphates in the vicinity of infected roots was demonstrated. The increase of soluble phosphates was correlated with greatly (*c.* 100%) increased growth and absorption of phosphorus and often earlier flowering in infected cultures provided iron and manganese were present in sufficient quantities. In iron-deficient media the presence of bacteria often caused a decrease of growth owing to the precipitation of iron by phosphates. Hence various degrees of stimulation or retardation of growth might be obtained by varying cultural conditions. Using virgin heath soil treated with basic slag Gerretsen demonstrated that inoculation with 1% garden soil increased the growth and phosphorus uptake of oats (by 72 and 49% respectively), so indicating a deficiency of the necessary bacteria in such soil. Treatment of this soil with superphosphate gave even greater growth increases when infected with bacteria. These results are so conclusive that great care must be exercised in interpretating results of experiments on mycorrhiza where inocula other than pure cultures were used to ensure the presence of mycorrhizal fungi.

Much work has been performed upon the effect of ectotrophic mycorrhiza-formers on their host species. A certain amount of disagreement and controversy between various workers has been evident because of the great difficulty in providing good control for any experimental series. This difficulty is especially acute when conditions approximating to the natural habitats are used. Certain of these difficulties have been foreshadowed above. For instance, there is undoubtedly a variation in the nature of the relationship between host and mycorrhiza-former with change of external conditions, and this is clearly so when there are morphological variations in the root system. Moreover, there are conditions when mycorrhiza formation is absent in well-grown young trees. Mitchell, Finn & Rosendahl (1937) have compared mycorrhizal and non-mycorrhizal seedlings of *Pinus strobus* under various conditions of growth. They showed that, on a heavily manured artificial soil of sand, sawdust and clay, seedlings with no mycorrhizas far exceeded mycorrhizal seedlings on the same substrate which was less heavily manured. Addoms (1937) has demonstrated that there is no significant difference of growth between mycorrhizal and non-mycorrhizal seedlings in soil relatively rich in nutrient. These and other experiments of the same sort make it clear that mycorrhiza formation is by no means obligate for the host plant and that there are experimental conditions at least where the effect of mycorrhiza on total growth rate may be negligible. It is essential not to put too much weight on this evidence. For, by ascribing the name mycorrhiza to an association between root and fungus, the investigators have assumed at least a morphological change of the root system of the infected plant, but few of them have described carefully the variation in morphology of the association under different experimental treatments.

The usual changes of morphology of the root after ectotrophic mycorrhizal infection are the following: (1) increase in diameter, (2) increase in number of cortical cells, (3) increase in size of cortical cells by hypertrophy, (4) increase in frequency of branching, (5) increase in surface of the mycorrhizal system due to the increase in the fungal sheath and hyphal connexions with the soil.

We have no certain knowledge of the mechanism by which changes (1) to (4) are brought about. Guesses could be made on the basis of what is known of the effects of growth substances on roots of whole plants and on excised roots, together with the results of work on abnormal and pathological growth (see, for instance, Grieve, 1943). Such guesses would not have a well-founded basis. Nevertheless, it seems certain that individual short roots infected with mycorrhizal fungi do not only increase in size and branching but also present an increased absorbing surface to the soil.

The increase in surface of typical mycorrhizal roots of *Pinus* forms a basis for the hypothesis of Hatch (1937) on the effects of ectotrophic infection. Recently Routien & Dawson (1943) showed that 1 in. long segments of the long roots of *Pinus echinata* produced similar numbers of short roots in infected and uninfected plants under their experimental conditions. The infected short roots were longer and much more frequently branched. This result, although not in agreement with the suggestion that there is an increase in the number of short roots in infected plants, corroborates Hatch's result of increased surface area in mycorrhizal roots themselves. Hence, where infection results in the formation of a sheath and intracellular penetration, there is a general increase in root surface area of individual roots. Where infection results in penetration of the cells, or in the incomplete development of the sheath or of the intracellular net, there may be no very significant increase of surface and there may develop, in addition, a cortical condition which can be construed as parasitic attack.

Such a conclusion does not justify without question the assumption that the total absorbing surface of the root system is increased in plants equipped with typical ectotrophic mycorrhizas. Because, if growth is increased by infection, such growth may offset the increase of root surface, owing to the increase of those organs not engaged in absorption from the soil. The only type of experiment which helps in solving this problem is that in which the only variable is the presence or absence of mycorrhiza fungi in the soil in which experimental seedlings are grown. Examples of such experiments are relatively few. Moreover, there is often no clear description of the mode of inoculation of the fungi, and sometimes there is evidence of the addition of a food base with the fungi which might contain essential material for the growth of the host. If such examples in which there is a considerable complication are eliminated, a few presumably satisfactory experiments are left, in which the results of infection are suitably expressed in terms of total growth and relative root growth. For instance, Hatch (1936) inoculated pine in pure cultures with several known mycorrhiza fungi and obtained the following average results:

	Dry weight (g.)	Root/shoot ratio	Nitrogen (%)	Phosphorus (%)	Potassium (%)
Mycorrhizal seedlings	404·6	0·78	1·24	0·20	0·74
Non-mycorrhizal seedlings	320·7	1·14	0·85	0·08	0·43

Here there is a reduction of relative root growth as measured by proportionate weight in mycorrhizal seedlings and, hence, a probability that the increase of surface of individual roots may be compensated by a decrease of total root growth in proportion to the growth of the other organs. Similar indications are given in the work of Finn (1942) and elsewhere; but in each case there was less control of experimental conditions.

Leaving aside toxicity of soil as a factor, it has already been mentioned above that relative root growth is inversely correlated with nutrient concentration over a considerable range. Hence the decrease of relative root growth in the inoculated plants may also be a reflexion of such an increase of supply which could be due, directly or indirectly, to the activities of mycorrhizal fungi or to materials introduced with the inoculum and modified by those fungi. The figures above confirm the fact that the quantities of nitrogen and phosphorus per unit dry weight present in mycorrhizal seedlings exceeded the quantities present in non-mycorrhizal seedlings. That is, a disproportionately greater absorption of these elements had taken place. Hence we may question the stress placed by Hatch upon the physical effect of the fungi in increasing the absorbing root surface, but it does appear that the total root growth and relative salt absorption was increased in these plants in which mycorrhizal fungi were present and in which synthetic organs were formed. Even allowing Hatch's further point, that there is a delay in the suberization of root tissues of mycorrhizas, it is clear that the physical increase of root surface may be considerably offset by the decrease in relative root growth.

In this experiment the total growth was increased by the presence of mycorrhizal fungi which apparently was the sole variable. Other experiments suggest the same increase of growth, but, in most of them, the presence of mycorrhiza fungi was determined by chance, or by the addition of soil, humus inocula or root fragments, or by the presence of an infected seedling amongst the experimental plants. Rayner (1938) and others have reviewed some of the relevant literature which seems to demonstrate that the formation of mycorrhizas or the presence of the fungi concerned does, under certain conditions, lead to the increased growth of the host species and sometimes also to decreased relative root growth. Complications are apparent in some experiments, such as those of Rayner on Wareham Forest soil, because of inimical factors arising out of the activities of the soil population. Again, the introduction, in some mixed inocula, of potential parasites has been shown to lead to a soil condition resulting in pathological states of the roots or of the mycorrhizas. Much has been achieved by the use of these crude inocula and by soil amendments in the sphere of practical forestry (see especially Rayner & Neilson Jones, 1944) but the results are not capable of detailed analysis, so that the mechanism or the mode of action of the associated organisms cannot clearly be seen. Young (1940*b*) inoculated nursery seedlings of *Pinus* with various species of fungi (contained in oatmeal and malt extract) and obtained mycorrhiza formation and variable dry weight increases of the seedlings in one year. The experiment was uncontrolled, so that we can only conclude from it that different fungi exerted a different effect on the host plant under similar external conditions, even though they all produced what were described as mycorrhizas. Here is offered an approach to the problem of the mechanisms by which the stimulation of growth is brought about, through a detailed study of the comparative physiology of the fungi, the comparative morphology of the mycorrhizas they form, and of a comparison of their effects upon soil organisms and processes.

VII. A SURVEY OF HYPOTHESES PUT FORWARD TO EXPLAIN MYCORRHIZAL PHENOMENA

At present we have to admit that the true nature and mechanism of the action of the mycorrhizal fungi upon their host is not at all clear. Hypotheses varying from the purely physical action of the fungi upon root-absorbing area, through views upon the acceleration and localization of general soil processes and specific reactions such as the increase of availability of nitrogen and phosphorus by the fungi, to the extreme suggestion of a mutual provision of growth-promoting substances, have been put forward by various workers. Whatever view one takes of the physiological significance of ectotrophic mycorrhizas from the evidence available, it is essential to appreciate that a high proportion of absorbed materials must enter through the fungal sheath of infected roots. It is unlikely that the living sheath can be dismissed as a kind of wick, but it must be considered as a living system with a certain power of selective absorption. Its surface of contact with the soil is that of the outgoing hyphae and the sheath, and its inner surface of contact with the root is the inner sheath surface, together with the intercellular hyphae. These intercellular hyphae must increase very materially the contact for transference of material, and this explains the stress laid by such workers as Rayner upon the significance of the morphological structure of the composite organ and upon variations of its efficiency with variations of structure. The intensity of the effect, beneficial or otherwise, of any particular association may well increase with the physiological activity of the fungal partner and the closeness of the association for the exchange of materials between the partners. Comment has already been passed on the view of Hatch that the importance of mycorrhizal infection lies in a physical increase of root surface. It is not possible to deny this view on present evidence. It has one particular merit, however, because it attempts to explain why some experimenters have stressed the effect of mycorrhizal infection in increasing the absorption of nitrogen, others of phosphorus and yet others of further soil compounds. A general increase of root surface might be expected to increase the ease of absorption by the roots, and so, if there is a degree of selection, to exert the greatest effect upon those essential compounds of lowest availability.

Other hypotheses take into consideration the special physiology of fungi, particularly the supposed property of mycorrhizal fungi of simplifying soil compounds by enzyme action. Burges (1936) suggested that the fungi concerned with mycorrhizas are weak pathogens whose activities are controlled by the host. He regarded the simplification and dissolution of soil materials as a separate and purely coincidental part of their activities, which might not always occur. His interest in endotrophic mycorrhizas seems to have led him to ignore the completeness and the probable significance of the sheath of ectotrophic mycorrhizas. His assumption seems to be that there is a local increase of availability of materials in the root region of all plants, and in particular of mycorrhizal plants. The results of Mitchell *et al.* (1937) indicate that there was no such increase in the neighbourhood of mycorrhizal, as compared with non-mycorrhizal, pine seedlings. This does not dismiss the possibility that such an increase exists, partly on account of their relatively crude sampling methods, and, more especially, because concentrations in the root region are the only resultant of competing processes. If the absorption rate is great, then the concentration may be kept low even if the production rate is also high. Moreover, the detailed rhizosphere studies described in a preceding section have confirmed the fact that there may be a measurable increase of rate of soil processes in the root region, and the problem is therefore to what extent these processes are increased or decreased, directly or indirectly, by the mycorrhizal fungi, and to what extent absorption by the roots is also affected by them.

Recently, Routien & Dawson (1943) have compared growth rates and certain aspects of the physiology of mycorrhizal and non-mycorrhizal plants. Their technique did not involve the exclusion of all micro-organisms other than the mycorrhizal fungi which were introduced in soil inocula, and there seems to have been some form of fungal infection of the 'non-mycorrhizal roots' (see their footnote, p. 441). Their results, nevertheless, show that growth as measured by dry weight increment was greater for mycorrhizal than for non-mycorrhizal plants, especially when grown in substrata where the colloids were not saturated with bases and where there were considerable quantities of adsorbed hydrogen ions. These results are of great interest because Hatch and others have shown that there is an increased degree of mycorrhizal development in base-unsaturated soils. The production of carbon dioxide by equal lengths of mycorrhizal and non-mycorrhizal long roots showed that the former had a higher aerobic and anaerobic rate of respiration. It might be expected from recent physiological work on salt absorption that the higher metabolic rates of mycorrhizal roots would be associated with higher absorption rates of inorganic ions. Routien & Dawson (1943) suggest that mycorrhizas increase the rate of salt absorption by increasing the supply of exchangeable hydrogen through greater release of carbon dioxide in respiration. These results showing respiratory increase are only really valid if we can accept the method of sampling the root systems on the basis of unit length. This involves the assumption that the relative linear extent of the root system of mycorrhizal and non-mycorrhizal plants is similar. Such may not be the case. The results of Routien & Dawson, nevertheless, conform with the knowledge of the effect of rhizosphere organisms on the availability of inorganic soil constituents, especially of phosphorus, and they provide further evidence that general rhizosphere effects and mycorrhiza are parts of one series of phenomena.

The hypotheses concerning the mutual stimulation of fungus and root by accessory food substances make use of only a part of the experimental results available. Enough is known of the sugar and vitamin demands of the mycorrhizal fungi to suggest some explanation of their association with roots, but not of their dominance in the root region, or of their penetration or formation of composite organs. Nor is any explanation yet available of the changes of form and hypertrophy of the roots themselves. The production of auxins, or auxin-like substances by fungi and other micro-organisms has been demonstrated, and although this property is known to be possessed by at least one basidiomycete (*Boletus edulis*) it brings us no nearer a solution of this problem than it does to the problem of crown-gall and other pathological growths. Lindquist (1939) has performed certain experiments with spruce seedlings by treating them with sterile solutions on which fungi had been grown. He obtained slight, but often apparently significant, differences in growth between the spruce plants so treated and those receiving the original culture fluid. He concluded that metabolic products of fungi capable of forming a mycorrhiza with spruce stimulated growth. The products of 'indifferent' fungi did not stimulate growth and those of parasitic fungi depressed growth. He therefore suggested that there was a mutual stimulation of mycorrhizal fungus and host by the by-products of each other's metabolism. The results are not sufficiently convincing for immediate acceptance, and, since neither the true mechanism nor the nature of the substance concerned is known, no particular weight can be placed on this hypothesis.*

Recently MacDougal & Dufrenoy (1940, 1941, 1943, 1944, 1946) have renewed interest in cytochemical staining methods for determination of the mutual exchange between root and fungi in mycorrhizas. Their most striking claim is that completely detached root segments bearing mycorrhizal short roots are capable of carrying on life and growth in soil for 28 months. If such a claim is well substantiated it implies that all the food material for the maintenance of such segments is derived from the soil, presumably by the activity and through the tissue of the living fungus. This view is reminiscent of that put forward by Young (1940*a*) on somewhat doubtful evidence in his work on the Fused Needle disease of pine. It implies that whatever substances are derived from the root by the fungus must be synthesized by that root from materials released to it by the fungus. It queries at once the ability of the fungi to flourish only on sugars or simple sources of carbon, unless these occur in sufficient quantity in the soil itself. The existence of such simple compounds might be expected if it can be assumed that other micro-organisms which produce them are present in the rhizosphere. Unfortunately we are not in possession of a detailed description of the conditions under which these root segments were grown, so that further consideration of this matter, which so complicates our present conceptions, must be postponed until an adequate examination has been made of these phenomena.

This brief survey of some of the recent hypotheses shows that, in spite of the diversity of views held, there is much less disagreement on factual grounds than might have been expected. The various observers differ in the relative importance they attach to each section of the experimental results. Most of the theories are not mutually exclusive, and that of Hatch, for instance, does not specifically exclude any other. The point on which agreement is most general is that the presence of the mycorrhizal fungi in the soil or on the host root surface promotes increased growth of the host plant under some conditions. The fact that manurial treatments may themselves bring about a stimulation of growth in the absence of the fungi cannot affect this conclusion. The mechanism of the stimulation of the host plant by mycorrhizal fungi, whether it be due to increase of root surface, to increased absorption rates, or to the availability of specific substances, is quite obscure.

Future work should not, however, treat mycorrhizal relationships as an isolated series of phenomena but should take into consideration the results obtained in the related study of rhizosphere populations and soil-borne pathogens. It may be recalled that similar stimulation of the host has been described for some non-mycorrhizal associations between roots and micro-organisms, and that mycorrhizal fungi may act as parasites or unspecialized associates under certain conditions. When all these manifestations of associated growth are considered, the illusory differences between mycorrhiza and pseudomycorrhiza are avoided and the subject viewed in the proper perspective.

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* Mr D. J. Finney, Reader in the Design and Analysis of Scientific Experiments at Oxford University, very kindly examined the published figures of Lindquist for me. He pointed out that, if we ignore certain complications perhaps due to possible position effects between the culture vessels, the results in general show statistical significance, such as might be explained by Lindquist's hypothesis.

VIII. SUMMARY

1. Micro-organisms are not evenly distributed throughout the soil, and different local soil variations and horizons are inhabited by populations differing in activity and number. The root regions of green plants constitute an important group of these microhabitats, and the population of the root regions of some plants have been examined experimentally. In the root region a population differing from the general soil microflora is found, and here increased rates of activity of certain soil processes, changing the availability of essential plant foods, have been described. Micro-organisms of many types are subject to this rhizosphere effect, and roots differing in age and in genetic origin exert different effects on various types of micro-organisms.

2. The phenomena examined in mycorrhizal studies can be grouped naturally with general rhizosphere and root region phenomena. They differ essentially only in the relative dominance of one or few particular members of the root flora, but in typical cases of ectotrophic mycorrhiza the presence of the dominant fungus is associated with morphological changes in the root. Part of the confusion in the presentation of experimental results, and in the theoretical discussion of these results, is due to lack of appreciation of the general and widespread nature of rhizosphere effects and their dependence upon the nature and physiological state of the roots.

3. Similar hypotheses, based on experimental results, have been put forward to explain both the general case of association of roots and micro-organisms and the special cases of ectotrophic mycorrhiza. The excretion of substances causing stimulation of micro-organisms, such as amino-acids and vitamins, of food materials such as sugar, and of inhibiting substances, have all been suggested. Root excretions and extracts have been shown to affect the growth both of members of the flora of the root region and of mycorrhiza fungi.

4. No explanation is at present available as to why there is intercellular penetration, morphological change and the formation of composite organs of root and fungus, in typical cases of ectotrophic mycorrhiza; nor is there again any explanation of the variability of such associations with soil conditions.

5. The effect of the rhizosphere flora on the growth of the host plant has been examined. There may be a stimulation of growth by non-mycorrhizal rhizosphere fungi and bacteria. Similar stimulation of growth by mycorrhizal fungi has frequently been claimed. A great majority of observers agree that under some conditions the growth of tree seedlings bearing mycorrhizal roots greatly surpasses that of those lacking these roots. Very few experiments on this point are, however, quite satisfactory, owing to the difficulty of providing adequate controls. These few, together with the large body of imperfect experiments, are sufficient to enforce the acceptance of the fact that mycorrhizal fungi frequently stimulate growth.

6. The completeness of the living fungal sheath, and its intimate connexion with the root cortex in fully developed mycorrhizas, must be a fundamental consideration in the elaboration of hypotheses to explain the effects of infection on the host. Many of the existing theories do not take this sufficiently into account, because variations of the

morphology of mycorrhizas between experimental treatments have frequently not been sufficiently described. The existing hypotheses are not all mutually exclusive, but cannot be further co-ordinated or extended except by means of experiments more perfectly controlled than those yet described. Moreover, the activities or organisms associated with the true mycorrhiza-former require examination. Recent reports of the growth of excised mycorrhiza roots cannot be fully accepted until the results have been confirmed.

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THE STRUCTURE OF THE STRIATED MUSCLE FIBRE

BY ROBERT BARER
Department of Human Anatomy, Oxford University

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I. INTRODUCTION

The structure of the striated muscle fibre has for many years been a subject of great interest to the biologist. The remarkable regularity and apparent simplicity of organization as compared with most other cells; the relative ease with which individual fibres can be separated and their physical and chemical properties studied; the existence of a useful index of viability, in the form of contractility—all these factors have led biologists to expect that the relationship between form and function could best be studied in striated muscle.

Perhaps the first important contribution to the subject was that of William Bowman (1840). In a remarkably comprehensive and beautifully illustrated paper he laid the foundations of many of our ideas regarding muscle structure. It is a chastening experience to turn to his paper and to realize that apart from the substitution of photographs for drawings, remarkably little real knowledge has been added to the straightforward microscopy of muscle. Following Bowman there ensued a period of great activity, lasting until the end of the nineteenth century, chiefly among continental workers. The names of Kölliker, Krause, Kühne, Engelmann, Rollet and Ranvier continually recur in the literature.

The chief contribution of this period was the recognition of the complexity of the striations. Numerous 'lines', 'disks' or 'membranes' were described and designated by various letters of the alphabet, or by the names of those who described them. Most of these details were observed at a very high magnification, at, or even beyond, the extreme limits of resolution of the ordinary optical microscope. As we shall see, though many of the observations often became the subject of bitter controversy and were sometimes scornfully dismissed as artefacts or sheer imagination, they have been brilliantly confirmed by the use of the electron microscope. Another important contribution of this period was the observation that muscle fibres are birefringent. One of the earliest observations of birefringence in living organisms was that of Goddard (1839), who examined the larva of *Corethra* by polarized light. The birefringence of muscle was studied in greater detail by Boeck (1921), Brücke (1858), who recognized that the muscle fibre is composed of alternating singly refracting and doubly refracting regions, and Rouget (1862), as well as by many later workers. These observations initiated a number of controversies which have not been settled to this day.

Relatively little interest seems to have been taken in the problem of muscle structure in the years between 1900 and 1930. The old observers had attacked the problem with such energy and in so many ways that there seemed relatively little left to do. The chief results of this period have been discussed by Heidenhain (1911) in his celebrated *Plasma und Zelle*, and by Jordan (1933), who himself contributed much important histological work, especially on insect muscle. However, the physiologists and biochemists were far from inactive during this time. The work of A. V. Hill, Meyerhof, and others provided an insight into the chemical, mechanical and thermal processes accompanying muscular contraction. Some of this work threw new light on muscle structure, or at least provided new facts with which theories of structure had to be correlated.

An important new stimulus to the investigation of muscle structure came in 1930 with the isolation from muscle of a protein (myosin) which exhibits strong birefringence of flow (von Muralt & Edsall, 1930). Here, at last, cytologists were provided with a possible means of explanation of some of the phenomena of birefringence in muscle fibres. Weber (1934 *a*, *b*, 1939 *a*, *b*) carried out an important comparison of the properties of myosin solutions and threads with those of muscle fibres, and von Muralt (1932), and Buchthal & Knappeis (1938), investigated the changes in birefringence in muscle fibres during contraction. Such work left no doubt that the properties of myosin provide the key to muscle structure and muscular contraction. Powerful confirmation of this came from X-ray diffraction work. Boehm & Weber (1932) showed that the X-ray diffraction patterns of living muscle and myosin are virtually identical, and this was confirmed and extended by Astbury, who has recently summarized his work in a Croonian lecture (1947).

The 'Myosin Era' which began in 1930 has seen many new and important discoveries concerning muscular contraction. Perhaps none has captured the imagination more than the observations of Engelhardt and his collaborators (Engelhardt & Ljubimova, 1939), who showed that myosin appeared to possess enzymic activity, and in fact seemed to be identical with adenosine triphosphatase. Even more remarkable was the fact that adenosine triphosphate is capable of inducing a reversible increase in extensibility in myosin threads. Soon afterwards J. Needham and his collaborators (Dainty, Kleinzeller, Lawrence, Miall, Needham, Needham & Shen, 1944) reported that adenosine triphosphate causes a reversible reduction in flow birefringence in myosin sols. There is good evidence that the dephosphorylation of adenosine triphosphate is one of the earliest changes in muscular activity, so that this demonstration of a link between chemical and mechanical changes is of great importance. These observations were carried a step further by the work of Szent-Györgyi and his pupils (Szent-Györgyi, 1940, 1945 *a*, *b*, 1947). They succeeded in producing *contractile* protein threads from muscle extracts.

The relationship of these results to the structure of the striated muscle fibre will be discussed later, but at this point it may be as well to state the object of this article. It is a tribute to the work of the physicists and chemists that the mention of the word 'muscle' conjures up in the minds of many people not so much a living cell as an X-ray diffraction pattern or a contractile protein thread. To a biologist, however, a muscle fibre is something more; it is a living cell, with all the properties peculiar to living matter. It would be a mistake to assume that the behaviour of a muscle fibre can be entirely explained by the properties of myosin alone, important though the role of the latter may be. The muscle fibre is a complex system containing cell membrane, nuclei, undifferentiated protoplasm, lipoids, salts and a host of complex chemical substances, including very many proteins besides myosin. The environment of myosin in such a complex organization may be very different from the relatively simple environment of the test-tube, and it is essential to interpret the results of *in vitro* experiments with great caution.

In this article I shall attempt to discuss what is known of the structure of the individual components of the striated muscle fibre, and to show how these components are interrelated to form the complete fibre. We shall not be primarily concerned with molecular structure except in so far as the work of the molecular physicists and chemists may throw light on microscopic structure. The view which will be taken as a basis for discussion is that the muscle fibre is composed of a large number of myofibrils embedded in a viscous matrix of undifferentiated protoplasm or sarcoplasm, containing numerous nuclei, the whole being enclosed in a fine sheath, the sarcolemma. Each fibre receives a nerve supply and is connected terminally to connective tissue and tendon fibres.

II. THE SARCOLEMMA

Considerable confusion exists regarding the nature of the investing membranes of the striated muscle fibre. In physiological literature reference is frequently made to a 'membrane' which is believed to be the site of ionic interchanges and potential differences. Such plasma membranes have been recognized and their properties determined in many cells (see Danielli, 1942; Chambers, 1940) but the exact localization of this membrane in the muscle fibre is a matter for conjecture. Gutmann & Young (1944) state: 'Presumably there is a cell membrane at the surface of the sarcoplasm which is responsible for maintaining the difference in concentration between the outside and the inside of the fibre, separating a fluid rich in Na^+ and Cl^- outside from the K^+ space within. This membrane must be located at the outer edge of the thin band of sarcoplasm mentioned above, but there is no reason to suppose that it is a thick, visible membrane. The layer which is usually called sarcolemma is quite a thick membrane which lies more peripherally, and probably does not strictly belong to the muscle fibre, but to the endomysium. There is no reason to suppose that this thicker layer has special permeability properties.'

The obvious comment which one might make on this statement is that while there may be no reason to suppose that the sarcolemma has any special permeability properties, there is equally no reason to suppose that it has not. In fact, very little is known of the structural, mechanical, and physiological properties of this important membrane. Indeed, there is considerable divergence of opinion as to what should properly be called the sarcolemma. Most of this confusion seems to have arisen from the study of fixed and stained sections. No such confusion existed in the mind of Bowman (1840) when he described 'a tubular membranaceous sheath of the most exquisite delicacy, investing every fasciculus from end to end, and isolating its fibrillae from all the surrounding structures'. It was Bowman who named this sheath *sarcolemma*, and there seems to be little necessity to deviate from his description.

The simplest method of demonstrating the sarcolemma is to examine a few roughly teased fresh striated muscle fibres in Ringer's fluid. As a rule, after a few minutes the muscle substance appears to clot at sites of injury. The striations seem to coalesce, forming a granular mass of debris known as a 'retraction clot' (a detailed description of the formation of retraction clots has been given by Speidel, 1939). As these clots form they retract, leaving an empty space within the sarcolemma and enabling the latter to be studied free from muscle substance. The sarcolemma can be revealed in many other ways. Anything which produces a violent contracture of the muscle fibre, such as treatment with caffeine, nicotine or saponin, may cause the sarcolemma to bulge away from the contracted muscle substance. Colloidal swelling agents such as dilute acids or alkalis produce swelling of the muscle substance, which may flow out of spontaneous ruptures of the sarcolemma (Barer, 1947*a*). The passage of a constant galvanic current along a short length of a muscle fibre often produces a granular disintegration of the muscle substance leaving a clear area of sarcolemma (Barer, unpublished). Treatment with trypsin digests the muscle substance, leaving the sarcolemma apparently intact. When examined by ordinary illumination after treatment by any of the above methods the sarcolemma appears to be an extremely thin homogeneous membrane. One may often observe a number of wavy fibrils which appear to be attached to the outside of the sarcolemma. These are probably the reticular fibres which are such a prominent feature in appropriately silver-stained sections of muscle. They are particularly well seen by phase-contrast illumination and do not appear to form an integral part of the sarcolemma proper but constitute part of the sheath of adventitious connective tissue, the endomysium, which surrounds the muscle fibre.

Long (1947) has recently defined the sarcolemma as a double structure consisting of an inner layer which is regarded as a plasma membrane and an outer part composed of delicate reticular fibres closely adherent to the inner layer. This description fits in well with the appearances seen in fresh teased muscle fibres. I prefer to restrict the term 'sarcolemma' to the thin, apparently structureless membrane, as described by Bowman. Much of the confusion seems to have arisen from the fact that, as Long (1947) points out, the membranous structure is too thin to be studied in ordinary fixed and stained preparations and it is the adventitious connective tissue element which has been designated as sarcolemma.

Most of the work on the sarcolemma has hitherto been carried out on stained material (Asai, 1914; Peterfi, 1913). Nagel (1935) and Bairata (1937) have used both stained and fresh material. They regard the sarcolemma as a meshwork of collagenous or reticular fibres embedded in a homogeneous colloidal matrix. Bairata (1937) claims to have demonstrated the presence of fine fibrils within the fresh sarcolemma. These fibrils could not be seen by ordinary illumination but were rendered visible by dark-ground illumination or by polarized light, when they appeared to be birefringent. Attempts have been made to repeat these observations but without success. The material used by Bairata consisted of pieces of muscle fibre digested with trypsin. This type of material has proved to be quite unsuitable for studying the structure of the sarcolemma in any detail, and I have found it impossible to make a clean preparation of the sarcolemma in this way. The muscle substance is very rarely completely digested by trypsin and there is nearly always a good deal of granular debris which tends to stick to the inner surface of the membrane. This granular debris is very evident in Bairata's dark-ground photograph; even the background is full of it. It is a very far cry indeed from Bairata's photograph to his beautiful diagram of interlacing fibrils. A similar criticism may be directed against his polarized light photograph. The granular residue left after tryptic digestion is usually birefringent and the true structure of the sarcolemma is obscured. Another objection to the use of trypsin is that it may partially digest the sarcolemma itself. The material used by me was mainly fresh teased muscle fibres of various species, allowed to soak in Ringer's solution for about ½–1 hr. As we have seen, if the teasing process is rather rough, retraction clots form, leaving clear spaces within the sarcolemmal sheath. Very little debris is left by this method. Other methods used included the use of contracture-producing drugs, passage of electric currents, and swelling agents, as mentioned above. Tryptic digestion followed by careful washing was also used. If care was taken to obtain a clean preparation, no evidence of any structure, fibrillar or otherwise, could be found when using ordinary, dark-ground, polarized light or phase-contrast illumination. The absence of fibrils in the phase-contrast method is particularly noteworthy as this method is as a rule extremely sensitive for demonstrating small fibrils. Reticular fibrils attached to, but not embedded within, the sarcolemma can be seen with great ease (Barer, 1948).

Since the usual methods of microscopy failed to reveal any structure, a preliminary study of the sarcolemma has been carried out with the electron microscope (Jones & Barer, 1947). This work is still in an early stage, but up to the present no fibrils have been seen. The only structures observed with moderate magnifications (up to 25,000 times) have been numerous small dots of the order of 0·04–0·1 μ in diameter.

These spots occasionally appeared to be arranged in a more or less regular pattern, the distance between them being of the order of 0·5 μ. In specimens in which the membrane has become folded on itself it can be seen that the spots appear as nodules raised above the surface of the membrane. The only clue to the nature of these spots is that they disappear after treatment with 3% citric acid. It is possible that they represent points of attachment of reticular fibres to the sarcolemma, but no actual fibres have yet been observed.

The electron microscope enables us to make an estimate of the thickness of the sarcolemma. It is generally stated that the membrane is about 1 μ thick. This figure is not based on any sound facts. Any very thin line which is beyond the resolving power of the miscroscope will appear as a diffraction line which to a casual examination seems to be about 1 μ thick. Similar lines in folded pieces of sarcolemma are seen with the electron microscope, but they are not more than 0·1 μ thick. Even this estimate may be much too high. At the same time, of course, some shrinkage of the membrane due to drying cannot be ruled out. It is interesting to note that if the thickness of the sarcolemma is accepted as being less than 0·1 μ, then so far as thickness is concerned it may very well be regarded as a plasma membrane. Unfortunately, measurements of cell membrane thickness have hitherto been very indirect and the results have varied within wide limits. Thus, calculations based on measurements of resistance give thicknesses between 1 and 0·001 μ. Waugh & Schmitt (1940), using an ingenious optical method, estimated the thickness of the rabbit red cell membrane at about 0·02 μ. If we take into consideration the great margin of error inherent in these measurements, the thickness of the sarcolemma is probably of a similar order of magnitude.

As regards the chemical nature of the sarcolemma very little is known. The claim that the membrane is composed of collagenous fibres is difficult to support on structural grounds, and experiments have shown that fairly pure collagenase preparations (from *Clostridium welchii* cultures) have no apparent effect on the structure of the sarcolemma (Barer, unpublished). It should be possible to obtain sufficient free membrane to carry out a thorough chemical analysis. Such an investigation would be very valuable, as hitherto the only cell membrane available for chemical analysis has been that of the erythrocyte (Parpart & Dziemian, 1940).

As regards the mechanical properties of the sarcolemma the evidence is somewhat conflicting. Ramsay & Street (1940) produced retraction clots in single muscle fibres and found that the empty sarcolemma showed the same response to stretching as the intact fibre, i.e. the same increase in load produced the same percentage elongation. This suggests that the resting tension of the muscle fibre was governed by the sarcolemma alone. It is interesting to recall that Banus & Zetlin (1938) found that if the connective tissue sheath of a whole muscle were dissected free, it gave the same tension-length curve as the whole muscle. Unfortunately, Ramsay & Street's results were not confirmed by Sichel (1941), who found that the elongation of the empty lengths of sarcolemma was on the average 2·2 times that of the intact regions. On this basis an intact muscle fibre should resist extension by a tension at least twice that of the sarcolemma alone. According to Buchthal (1942) the sarcolemma in the region of a retraction clot is already in a state of elongation, so that the true resting tension is not obtained. This appears to be true where a large hard clot is formed, but this is not always the case. It is possible to produce empty lengths of sarcolemma without such clots and without any apparent lengthening. A reinvestigation of the mechanical properties of the sarcolemma is highly desirable, as the sarcolemma must perform an important role in muscular contraction. In particular, the elasticity of the sarcolemma may be sufficient to account for the appearance of 'active' relaxation in single muscle fibres (see Fenn, 1945).

III. THE SARCOPLASM

Of all the constituents of the striated muscle fibre the sarcoplasm is perhaps the least studied and least understood. The term is generally taken to refer to interstitial substance in which the myofibrils are embedded. The sarcoplasm is usually regarded as semifluid undifferentiated protoplasm. It is often very difficult to demonstrate—indeed Hürthle & Wachholder (1925) describe it as 'a schematic abstraction'—but its presence can usually be inferred from an examination of transverse sections of muscle fibres. The myofibrils can then be seen not to fill the entire space provided by the sarcolemma, the deficit being presumably filled by sarcoplasm.

The relative amount of sarcoplasm evidently varies considerably in the fibres of different animals. According to Buchthal (quoted by Krogh, 1947) about 40% of the volume of the frog's muscle fibre may be occupied by sarcoplasm. The proportion of sarcoplasm is even greater in some muscles. Rollet's classic diagram of a transverse section through the fin muscles of the sea horse (*Hippocampus*) is an excellent example of a muscle rich in sarcoplasm (Heidenhain, 1911). The distribution of sarcoplasm in muscles of different animals was exhaustively investigated by Knoll (1891), who divided muscles into a protoplasm-rich and a protoplasm-poor category. The former usually appear somewhat darker than the latter, and it is unfortunate that these two types are often regarded as identical with red and white muscle fibres respectively. This is very far from being the case. The colour of a muscle fibre depends mainly on its content of muscle haemoglobin (see Needham, 1926; Millikan, 1939), not on the amount of sarcoplasm. Thus, although it is perhaps true to say that red fibres tend on the whole to contain more sarcoplasm than white fibres, this statement is by no means universally true. One frequently finds red fibres which contain very little sarcoplasm, and white fibres which contain a high proportion. Other structural differences which have been described, such as the larger diameter of white fibres, and the more peripheral distribution of their nuclei, are equally unreliable as criteria.

Granules. Many observers have described the presence of granules in the sarcoplasm. These granules were studied by Kölliker (1856), who found both fatty droplets and small interstitial granules which were resistant to caustic alkalis and acetic acid. Bullard (1912) carried out a careful study of these granules and was able to confirm Kölliker's findings. He came to the conclusion that the small interstitial granules were of a protein-lipoid complex nature, probably identical with the mitochondria. He was unable to determine the exact nature of the larger fat droplets but could find no evidence to show that they contained anything other than neutral fat. Both Bullard (1912) and Bell (1911) claimed that the number of fat droplets varies with the nutritional state of the animal and can be regarded as a reserve foodstuff. The important work of Dempsey, Wislocki & Singer (1946) on the distribution of lipoids in the muscle fibre will be referred to later.

Glycogen. It is usually stated that glycogen occurs mainly in the sarcoplasm. Arnold (1909), using both Best's carmine and iodine, concluded that glycogen is entirely absent from the myofibrils, but can be demonstrated in the sarcoplasm mainly adjacent to the *I* bands. According to Studnitz (1935), however, glycogen occurs both in the sarcoplasm and in the myofibrils. He claimed that in the resting frog sartorius glycogen is mainly distributed in relation to the *A* bands, in contradistinction to Arnold's findings. The localization in the *A* bands was even more marked in muscles fixed after stimulation. During recovery, however, glycogen first appeared in relation to the *I* bands, later in the *A* bands. Studnitz suggested that the lactic acid is produced in the *A* region during contraction, diffuses into the *I* region and is resynthesized to glycogen, which finally returns to the *A* region.

The recent work of Dempsey *et al.* (1946) has not clarified the issue. They write: 'Glycogen is ordinarily described as occurring in scattered granules within the substance of myofibrils. Our results are entirely in accord with this statement.' I have been unable to find any reference to glycogen being found entirely in the myofibrils. It is usually regarded as a sarcoplasmic constituent. Dempsey *et al.* did not find any very regular distribution of glycogen in relation to the striations. It is clear that the question requires further study. Some of the contradictory statements are no doubt due to the use of poor methods of fixation and non-specific staining methods. Dempsey *et al.* admit that the two methods they used (Bauer's method and an ammoniacal silver method) are not entirely specific. The extent to which glycogen may be altered by fixation or the extent to which it may diffuse during the process of fixation may be another factor which has not yet received sufficient attention.

Proteins. It is unfortunately not possible to locate any specific proteins with certainty in the sarcoplasm. According to Weber (1934*a*, *b*, 1939*a*, *b*) myogen, the albumen fraction of the proteins isolated from whole muscle, is confined to the sarcoplasm. The evidence for this statement is rather negative. The solubility of myogen is greater than would correspond to the concentrations that are believed to exist in muscle. The inference is that it must exist in solution and can take no part in the structural elements of the muscle fibre. This only leaves it the sarcoplasm in which to reside. This type of evidence is very indirect and furthermore makes the rather dangerous assumption that conditions within the living fibre must be the same as *in vitro*. For want of any better evidence we may accept it temporarily but with an open mind.

Even among biochemists there is no general agreement as to the protein constituents of the sarcoplasm. Thus Weber (1939*b*) states that globulin *X*, the chief globulin component of muscle press juice, is rather insoluble and 'the greater part of it must form structures in the living muscle'. Smith (1937), on the other hand, states the contrary: 'It seems quite likely, however, that in living muscle at pH 7·3–7·5 the whole of the globulin *X* would be soluble in the juice.' According to him the sarcoplasm contains myogen, myoalbumin and globulin *X*. The function of these proteins is uncertain at present. Interesting progress has been made recently in the purification of the myogen fraction. Thus Baranowski (1939) was able to separate two crystalline proteins from rabbit myogen. He called these myogen *A* and myogen *B*. According to Engelhardt (1942) myogen *A* possesses aldolase-zymohexase activity. Herbert, Gordon, Subrahmanyan & Green (1940) have isolated another fraction from myogen which is apparently pure zymohexase. As they point out: 'There can be no question therefore that what have hitherto been regarded as the main proteins of skeletal muscle are merely mixtures of enzymes (or other proteins) with superficially similar properties.' Caputto & Dixon (1945) have also isolated a triose phosphate dehydrogenase which they believe may be identical with myogen *B*. The immediate interest of such observations lies in the fact that the myogen fraction appears to be associated with various enzymic activities. If we can accept the evidence that the myogen is confined to the sarcoplasm it is likely that the latter may play an important metabolic role. Such a role has frequently been suggested (Embden & Lawaczeck, 1923; Embden & Lange, 1923) but the evidence has been rather indirect. It is to be hoped that sensitive methods for the exact localization of enzymes within cells may help to solve the problem.

Sarcoplasmic contractility. It is generally believed at the present time that the myofibrils are the only contractile elements in the muscle fibre, the sarcoplasm playing a passive role. It is remarkable to note that no less an authority than Kühne, in his Croonian lecture (1888), expressed the belief that the myofibrils are purely elastic elements, the sarcoplasm being contractile. Botazzi (1897) supported the idea of sarcoplasmic contractility, but believed that the myofibrils are also contractile. From observations on the variations in rhythmic contractions in the auricle of the tortoise he concluded that the sarcoplasm is responsible for slow tonic contraction. This idea was later extended to skeletal muscle. From a study of the electrical responses of normal and degenerating red and white muscles, Roberts (1916) also concluded that the sarcoplasm is capable of contraction. He suggested that in a normal white muscle the myofibrils alone respond to stimulation. During degeneration the excitability of the myofibrils diminishes while that of the sarcoplasm increases; this would explain the alterations in response to various types of electrical stimuli.

It is most unfortunate that the problem of sarcoplasmic contractility has become confused with that of muscle tone. It is one thing to suggest that the sarcoplasm is contractile but quite another to suggest that it is responsible for tone or any other specialized form of contraction. The situation was made even more confused by the claims of some workers (de Boer, 1921; Boeke, 1921) that the sarcoplasm receives a special sympathetic nerve supply. This in turn associated the sarcoplasmic contractility problem with the controversy over the influence of the sympathetic nervous system on muscle tone (see Cobb, 1925; Hunter, 1924; Hinsey, 1934). In view of the general confusion of ideas on the sarcoplasm, muscle tone and sympathetic innervation of skeletal muscle, it is perhaps not surprising that the fundamental problem has become submerged. Thus Fulton (1926) states that 'the hypothesis [of sarcoplasmic contractility by Barer] appears to be unnecessary to account for the phenomenon which it was devised to explain, namely, the maintenance of posture of skeletal muscle'. We are not concerned with an hypothesis devised to explain tone, but with the more fundamental question of whether the sarcoplasm can contract. It is curious that biologists have for years argued about the problem in a most indirect manner. It is difficult to see how one could hope to solve the problem except by direct observation. Neither physiological records of muscular contractions nor examination of fixed and stained tissues can give more than indirect evidence.

Direct observations on skeletal and cardiac muscle cells grown in tissue culture undoubtedly support the idea of sarcoplasmic contractility. M. R. Lewis (1915) and W. H. & M. R. Lewis (1917) first reported that unstriated myoblasts from skeletal muscle sometimes exhibit spontaneous rhythmic contractions. Friedheim (1931), however, claimed that contractility cannot occur before some degree of cross-striation is present. This cross-striation could not be demonstrated by ordinary methods but only by the use of monochromatic polarized light. The subject was investigated in some detail by de Renyi & Hogue (1934, 1938). They showed that myoblasts from skeletal muscle respond to direct mechanical stimulation with microneedles, at a stage when no striation can be detected by ordinary methods or by polarized light. Contraction takes place after a long latent period, lasts up to about 12 sec., and is followed by a slow relaxation. In some cases contractions lasting many hours were seen. The physical state of the sarcoplasm was found to differ in the relaxed and contracted states. Thus the sarcoplasm of a contracted fibre liquefies more slowly after injury and is more resistant to pressure and pull applied with microneedles. The same workers obtained equally interesting results with tissue cultures of heart muscle. They observed both fibrillated and non-fibrillated cells and found that the mode of contraction is similar in each case. In cells containing both fibrillated and non-fibrillated regions, neither region acts independently. The non-fibrillated region becomes shorter and thicker. The fibrillae shorten but remain straight. It is noteworthy that these workers considered it necessary to prove that the myofibrils are contractile. This they did by injuring the sarcoplasm, leaving the fibrillar portion of the cell relatively intact. The latter continued to contract rhythmically though at a slower rate than normal. On the whole one is led to accept de Renyi & Hogue's (1938) statement that: 'Tissue culture material offers no evidence for the theory that myofibrillae when once formed are the only contractile elements. The sarcoplasm continues its contractile function undisturbed by the presence of myofibrillae.'

The work of Olivo, Petralia & Ricamo (1946) is of some importance in this connexion. They succeeded in recording the electrocardiogram of the chick embryo, both in intact embryos and in isolated cardiac primordia. Records taken at a very early stage, before the development of myofibrils, show only simple slow potentials. With the development of myofibrils a fast component appears, preceding the slow waves. Explants cultivated *in vitro* for many days undergo a progressive dedifferentiation with loss of myofibrillae. The electrical records from such explants show a corresponding return to the primitive pattern.

Evidence from direct observation on muscle fibres in the intact organism is unfortunately scanty. Speidel (1937, 1939) carried out an important series of observations on the intact fibres of the tadpole's tail. He found that unstriated myoblasts or plasmodia may occasionally exhibit simple contractions when under the influence of longitudinal traction. In fully striated fibres it is usually difficult to make any accurate observations on the sarcoplasm as the latter is commonly rather scanty. In a few cases Speidel was able to see fibres with fair amounts of sarcoplasm and he remarks that occasionally the sarcoplasm appears to take an active part in contraction. I have recently carried out a number of observations on muscular contraction in living, intact insect larvae, using phase-contrast microscopy (Barer, 1947*c*, and unpublished observations). This method gives a very clear picture of refractile granules and nuclei within the sarcoplasm. The muscles of certain insect larvae (*Chaoborus*, *Chironomus*) are composed of single fibres only and the process of muscular contraction is relatively easy to follow. The sarcoplasm is usually abundant. Such observations are not conclusive owing to difficulties in interpretation, but they certainly do not exclude the possibility of sarcoplasmic contractility. In all cases the sarcoplasmic granules undergo a jelly-like quivering, as has been noted by Speidel in tadpole's muscle, and in many cases the sarcoplasm appears to undergo an active shortening. This is particularly so when the contraction is of long duration.

Thus we see that, so far as concerns primitive muscle fibres—myoblasts in tissue culture and cross-striated fibres in amphibia and insect larvae—sarcoplasmic contractility is more than a mere theory. When we review both the old (Engelmann, 1875; Biedermann, 1896) and modern (Seifriz, 1942) evidence concerning the contractility of undifferentiated protoplasm, we must agree with Fenn (1945) that nature does not always choose the most obvious way of doing things. As he suggests, there is no reason why the sarcoplasm itself should not contain scattered myosin chains.

The question of sarcoplasmic contractility is far from settled. Although there are good indications that it may occur in rather primitive and larval muscle cells, direct evidence is lacking in the case of fully developed adult vertebrate muscle fibres. Nevertheless, one cannot agree with the statement of Buchthal & Lindhard (1939): 'The suggestion that the sarcoplasm possesses contractile properties is so far removed from facts of histology or physiology that it need not be discussed.' I can only repeat that a single unequivocal direct observation would be worth more than all the facts of histology or physiology put together.

IV. THE MYOFIBRILS

Although Bowman (1840) observed that it is sometimes possible to split single muscle fibres longitudinally into fibrils, he was not specially impressed by this fact, and it was left to Kölliker (1851) to suggest that such fibrils are the ultimate contractile elements. This view is nowadays accepted by most workers but there is still an occasional tendency to regard the myofibrils as artefacts. Thus W. H. & M. R. Lewis (1917) were unable to see myofibrils in living muscle cells in tissue culture, though they could be demonstrated after fixation. It is, therefore, worth while examining the evidence in favour of the existence of the myofibrils.

(1) A longitudinal striation is often seen in fixed and stained histological sections of striated muscle. Small groups of fibrils often tend to get splayed out at the ends of a fibre during sectioning (see Clark, 1945, fig. 44).

(2) In transverse sections of whole muscle fibres the muscle substance does not appear uniformly arranged, but as a mosaic of small areas, which would correspond to transverse sections of groups of myofibrils. (As we have already seen, the material filling up the spaces between these columns of myofibrils is the sarcoplasm.) In mammalian fibres these columns are fairly regular and polygonal in appearance; they are known as Cohnheim's areas.

(3) Single muscle fibres can often be dissociated into smaller longitudinal fibrils or groups of fibrils. Many of the older workers performed such operations by hand, using fine needles. More recently attempts have been made to demonstrate the myofibrils by microdissection. Chambers (quoted by Speidel, 1939, p. 510) could only peel off groups of fibrils of variable diameter and was apparently unable to demonstrate the myofibrils as individual units. Ensinger (1938) claimed to be able to demonstrate myofibrils by microdissection, but his description was very brief and no illustrations were given. I have attempted to repeat these results (Barer, 1947*a* and unpublished observations). The great difficulty about microdissection of the living muscle fibre is that the fibre tends to react to injury by formation of retraction clots (Speidel, 1939) whereby there is a local loss of structure. It is, therefore, very difficult to pull out myofibrils from the living fibre, though this has been done in favourable cases. Again, the sarcoplasm is very sticky in the living fibre so that the myofibrils tend to join together in irregular bundles. In the dead fibre, however, the process of microdissection is much easier, and striated myofibrils can be separated from such material as reconstituted frozen-dried fibres, acid swollen fibres, and thin frozen sections of fresh unfixed muscle. In the case of the frog such myofibrils are usually of the order of 1–3 μ in diameter, agreeing with the classical text-book description.

(4) Fine fibrils corresponding to myofibrils can be prepared by mechanical dissociation methods. Hall, Jakus & Schmitt (1946) prepared such fibrils for electron

microscopy by subjecting small pieces of formalin-fixed muscle to mechanical agitation. They found that the width of such fibrils obtained from frog's muscles varies between 0·2 and 3·0μ, most of them lying between 0·5 and 1·0μ. These figures are again in good agreement with those obtained by classical histological methods.

(5) The freezing experiments of Chambers & Hale (1932) provide interesting presumptive evidence for the existence of myofibrils. They observed the process of freezing of muscle fibres under the microscope and found that a number of slender longitudinal columns of ice advanced along the interior of the fibre. When the fibre was twisted before freezing the ice columns ran spirally. They interpreted these results as due to freezing of the sarcoplasm surrounding the longitudinally arranged myofibrils.

(6) Certain physical measurements on living isolated fibres point to the existence of regular longitudinal structures. Thus Buchthal & Knappeis (1940) observed intensity variations in the diffraction spectra obtained from living frog's fibres, which were interpreted as due to longitudinal fibril bundles about 6–8μ in diameter. They suggested that these are the equivalent of Cohnheim's areas. In a fibre of 100μ diameter there would be 80 to 90 such fibril bundles, with about 5μ thickness of sarcoplasm intervening between adjacent bundles. The same workers (Buchthal & Knappeis, 1938) also interpreted certain variations in the interference fringes observed during measurements on the birefringence of living muscle fibres as being due to a longitudinal fibrillar structure.

(7) Direct observation of living muscle fibres has been somewhat contradictory. On the one hand W. H. & M. R. Lewis (1917), as we have already mentioned, and Goss (1933) were unable to detect myofibrils in living tissue cultures. On the other hand, Jordan (1933) writes: 'In proof of the actual existence of fibrils and cross-striations [disks] I would offer first a personal observation under high magnification of striped muscle in the tail of young frog and salamander tadpoles. One who takes the trouble to have this experience will have no further doubt that fibrils and cross stripes are real structures in living muscle.' Speidel (1939) too is convinced that myofibrils exist in living fibres; they are readily seen in irritated fibres and are capable of contraction. Their average diameter in the frog tadpole is about 0·5μ. I am in complete agreement with both Jordan and Speidel on this point.

(8) It appeared to me that the most definite evidence in favour of the existence of myofibrils would result from experiments in which single myofibrils or groups of myofibrils could be made to contract independently. A preliminary description of such observations has already been given (Barer, 1947*b*).

(*a*) Attempts were made to produce contraction of myofibrils by electrical stimulation. It is well known that stimulation of isolated single muscle fibres using micro-electrodes may result in localized contractions (Pratt, 1930; Gelfan, 1933). I stimulated isolated frog's fibres by means of two non-polarizable micro-electrodes placed transversely to the fibre. A constant galvanic current of the order of 50–70μA. was used. While the current was being passed it was observed that the myofibrils on the side of the fibre near the positive electrode were in the contracted state. Those near the negative electrode were relaxed. As a result the fibre took up a curved appearance, with the concavity towards the positive pole. If the current was suddenly reversed a rapid alteration in shape took place, so that the concavity was once more in the direction of the new positive pole. These effects are remarkably reminiscent of those which can be observed in amoeba and other protozoa under similar conditions. Verworn (1899) described many similar effects in such material and refers to an 'excitation of contraction' at the anode accompanied by an 'excitation of expansion' at the cathode. If a current of sufficient strength and duration was passed, the organism would undergo a granular disintegration. I observed a very similar granular disintegration in muscle fibres under like conditions. The effect differs from retraction clotting, but the final result is to leave a length of empty sarcolemmal sheath between the two electrodes. These results were of interest in showing that a muscle fibre need not contract simultaneously throughout its entire thickness. This fact is very simply explained if the myofibrils are accepted as independent contractile elements.

(*b*) More convincing evidence was obtained when it was found possible to produce asynchronous rhythmic contractions of myofibrils, or groups of myofibrils, in isolated single muscle fibres. This phenomenon was first observed in the course of experiments on the effect of alkalis on muscle fibres, when it was noticed that slightly alkaline Ringer's solution occasionally induced a repetitive twitching in single fibres. On examination under the high power of the microscope the fibre was seen not to be contracting as a whole, but apparently small groups of myofibrils were undergoing a series of rhythmic contractions, out of step with each other. As a result the regular cross-striated pattern of the fibre was lost, i.e. the striations were well preserved within any given group of fibrils. Now, it is well known since the time of Biedermann that certain substances may evoke rhythmic contractions in skeletal muscle. Biedermann himself (1896) noted that a frog's sartorius muscle would beat rhythmically when immersed in a solution containing 5 g. NaCl, 2 g. Na_2HPO_2 and 0·4 g. Na_2CO_3 per litre (Biedermann's solution). Loeb (1905) extended this work and showed that many ions would evoke similar effects. It seems probable that the asynchronous rhythmic contractions of myofibrils in single fibres are due to the same or similar causes as the rhythmic contractions of whole muscles. However, the phenomenon has proved most difficult to investigate as it is very elusive and cannot easily be repeated at will on single fibres. Nevertheless, I have observed the effect with most of the substances listed by Loeb, and those who have seen it are left in no doubt of the existence of independent contractility in myofibrils.

(*c*) The demonstration of an asynchronous contraction of myofibrils is of interest in that it focuses attention on the fact that, during more normal types of contraction, all the myofibrils appear to contract synchronously, so that the cross-striated pattern is not affected. We may therefore suppose that some sort of synchronizing mechanism exists within the fibre, whereby all the myofibrils are induced to contract and relax together. The nature of such a mechanism is at the moment purely speculative. It may perhaps be that the motor end-plate is so constructed that the excitatory process reaches all the myofibrils simultaneously, or possibly the sarcoplasm has special conducting properties. Direct anatomical or physiological evidence on these matters is lacking, but such evidence as there is suggests that the motor end-plate is not concerned with synchronization (Katz, personal communication, 1947).

I have studied an interesting example of both myofibrillar contraction and synchronization in the living larvae of the midge, *Chironomus*. The posterior proleg muscles of this animal show a remarkable type of branching. Each muscle is composed of a single fibre only, which as it approaches the body branches into two. The lateral branch remains on the same side of the body as the proleg from which it originated, but the medial branch crosses over to the opposite side. The result is a model in muscle of the hemi-decussation seen in the mammalian optic chiasma (Fig. 1 *a*). Under certain circumstances, when the muscles are irritated, contraction waves are seen to pass along the fibres. These are particularly well seen under polarized light, when a highly birefringent bulge travels like a wave down the fibres.

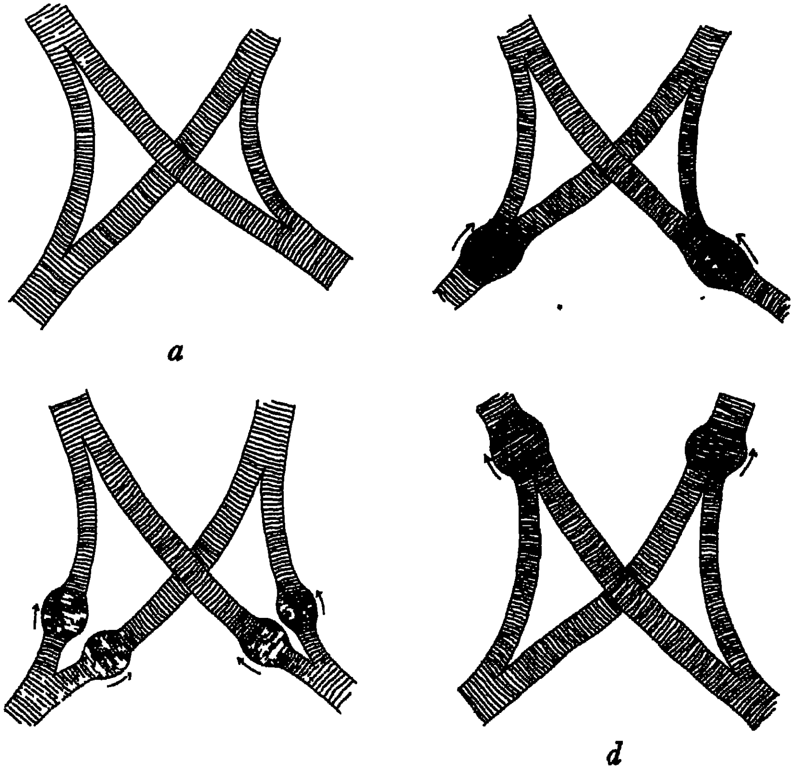

Fig. 1 *a*. Diagram showing arrangement of certain proleg muscles of *Chironomus* larvae; *b*, two contraction waves travelling along the fibres from the prolegs towards the body; *c*, splitting of each contraction wave at the site of branching of the fibres; *d*, fusion of contralateral branched waves.

Two such waves are depicted in Fig. 1*b* travelling from the prolegs towards the body. As these waves reach the branching region of the fibres they split into two (Fig. 1*c*). These waves travel along the branches until the latter reunite. As shown in Fig. 1*d* the wave in the lateral track from either proleg then unites with that in the medial (crossed) branch from the other proleg, the two smaller waves fusing into one large wave on each side. These observations suggest that the contraction wave is the result of the summation of synchronized waves in all the myofibrils. Occasionally a contraction wave originates on one side only. In this case the two branched waves continue as before but it can be seen that when the fibres reunite the wave passes down only one side of the fibre. This could hardly be the case if myofibrils did not exist. The fact that the two branched waves from opposite sides run into one perfect wave is an interesting example of synchronization. It may be that this synchronization is purely the result of the branched waves all having equal lengths of muscle fibre to traverse, but this does not always seem to be the case.

We must conclude, therefore, that there is very good evidence for the existence of myofibrils as independent contractile units in the living fibre. We shall now consider the structure of these units in more detail.

V. THE STRUCTURE OF THE MYOFIBRIL

Terminology. As we have seen, it was soon recognized that the cross-striations in many muscles were exceedingly complicated. A large and confusing literature arose in which new striations were described in the fibres of various animals. As a result the same striation is often designated by as many as four different names or letters of the alphabet. The details of this nomenclature have been given by Biedermann (1896), Heidenhain (1911) and Jordan (1933) and will not be discussed further in this article. In my opinion it is high time that our nomenclature be simplified and terms of purely historical interest excluded.

A certain uniformity of terminology has begun to appear in recent years among many workers on muscle structure. Two main striations can be recognized in the living or fixed muscle fibre. One of these is clearly birefringent when examined by polarized light. It is, therefore, designated the *A* or *Anisotropic* band. The other striation is generally said to be non-birefringent, though Schmidt (1935) has claimed that it is actually weakly birefringent. Nevertheless, the difference is sufficiently great to justify our calling this striation the *I* or *Isotropic* band. The *A* band usually appears darker than the *I* band when viewed with ordinary light. The mechanical properties of these two bands have been investigated in living isolated fibres by Buchthal, Knappeis & Lindhard (1936). They recorded changes during contraction by using a very intense flashlight lamp which enabled them to use short exposures for photography. In the resting fibre the *A* band averages $1\cdot37\mu$ in width, the *I* band only $0\cdot81\mu$. During isometric contraction the corresponding measurements are $1\cdot13$ and $1\cdot05\mu$. It will be observed that the total width of the $A+I$ bands remains constant at $2\cdot18\mu$. The *A* band shortens by 18%, whereas the *I* band lengthens by 28%. This is not to be regarded as showing that the *A* band alone is contractile. There is good evidence that the *I* band shortens in strong isotonic contraction. It seems likely that the contraction of the *I* band is usually masked by the more active contraction of the *A* band. The two bands also react differently to passive stretching. When the entire fibre is stretched by 40%, the *A* band stretches 51·8%, the *I* band only 23·5%.

Buchthal (1942) has more recently carried out a very detailed survey of the static and dynamic physical properties of the *A* and *I* bands, and Buchthal & Knappeis (1943 *a*, *b*), and Buchthal & Kaiser (1944) have correlated the changes in the *A* and *I* bands with the development of tension and propagation of contraction in single muscle fibres. Despite certain distinctions in optical and mechanical properties, Buchthal concluded that both disks take an active part in contraction and are composed of essentially the same substance, the differences being explained as due to differences of molecular orientation within the bands.

Apart from the *A* and *I* bands the only striation that can be recognized with any real certainty in the living fibre is the *Z* disk. This is a narrow dark band which bisects the *I* region. Its presence in living fibres has been confirmed by Speidel (1939), Buchthal & Lindhard (1939) and others. While it is true, as Bernal (1937) has remarked, that the optical appearance of muscle in ordinary light is delusive, there is no reason to believe that the *Z* disk is an optical artefact. Although other striations have from time to time been described in living fibres, particularly in insects, the difficulties in interpretation inherent in the examination of such material at very high magnifications are too great to allow us to place much reliance on such claims.

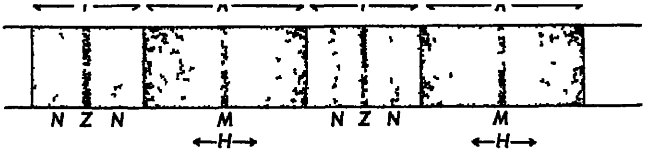

Fig. 2. Diagram of main striations seen in the single myofibril (rabbit). Based on the electron microscope photographs of Hall, Jakus & Schmitt (1946).

The evidence from fixed and stained sections is perhaps more reliable, but is still open to criticism. However, the recent electron microscope pictures of Hall *et al.* (1946) are so convincing that, while it must be remembered that fixed tissue was used, and that the possibility of alteration by the electron beam cannot be excluded, I feel that they should be taken as the basis for all future discussion of muscle structure. These workers used rabbit or frog muscles, fixed in 10% formalin, cut into small pieces and broken down into myofibrils by mechanical agitation. When viewed by the electron microscope these fibrils showed a number of striations. These are shown in Fig. 2, which is based on the electron microscope photographs. The *A* and *I* bands are clearly seen, with a dense *Z* band bisecting the *I* band. The length of myofibril bounded by two *Z* bands ($\frac{1}{2}I+A+\frac{1}{2}I$) is known as a *sarcomere*.

A narrow dark band may be seen bisecting the *A* band. This corresponds to the *M* band, or mesophragma, of the older histologists. A lighter region can often be recognized within the *A* band. This is the *H* (Hensen's) disk; it does not always appear to be present. Another inconstant feature is the presence of two dark (*N*) striations within the *I* band, one on each side of the *Z* band. These are found in rabbit muscles but are faint or absent in frog muscles. Finally, rabbit material may show two to four very fine striae close to the *M* band. These are not depicted in Fig. 2. The photographs on which Fig. 2 is based constitute a landmark in the study of muscle structure. Although photographs of muscle sections taken with the electron microscope have appeared before (Richards, Anderson & Hance, 1942; Sjöstrand, 1943), these are the first on which any real reliance may be placed. It is strongly to be hoped that the confusing terminology and fanciful diagrams, drawn at the utmost limits of both the resolving power of the microscope and the observer's imagination, will disappear from our text-books, to be replaced by electron microscope photographs or diagrams based upon them.

The work of Hall *et al.* (1946) has also thrown some light on the changes which occur during muscular contraction. In myofibrils from muscles stimulated to contract while held in a state of extension, the appearance of the light *H* band is very marked. On the other hand, the *H* band is faint or absent in fibrils from very strongly contracted muscles. The *I* band is very narrow in such myofibrils. At first sight this appears to contradict the observations of Buchthal *et al.* (1936) which have been described above. It must be remembered, however, that Buchthal *et al.* observed living fibres undergoing isometric contraction, whereas Hall *et al.* (1946) used muscles fixed during isotonic contraction. Another unknown factor is how far electron-scattering regions correspond to light-absorbing regions. It is not impossible that the apparent gross shortening of the *I* band seen with the electron microscope may in fact be due to the diffusion during contraction of some intensely electron-scattering material from the *A* band.

VI. THEORIES OF MYOFIBRILLAR STRUCTURE

At this stage it will be convenient to discuss some of the theories which have been put forward to explain the presence of the main cross-striations.

The A *and* I *disks are composed of different substances.* Theories based on this hypothesis were among the earliest to be postulated. Long before there was any accurate knowledge of the chemical composition of muscle it was common to speak of an '*A* substance' and an '*I* substance'. With the isolation of myosin and the demonstration of its flow birefringence (von Muralt & Edsall, 1930; Edsall & Mehl, 1940) it was tempting to regard the anisotropy of the *A* bands as due to the presence of myosin. Weber (1934*a*, *b*) put forward such a theory, in which it was suggested that the *A* bands were composed of myosin, the *I* bands of some other protein. Weber's calculations were based on analyses of rabbit muscles in which myosin formed only 39% of the total proteins (Meyer & Weber, 1933). Later workers have found much higher myosin contents. According to Smith (1937) 57% of the total protein of rabbit muscle is composed of myosin. Even higher proportions were found in fish muscles—up to 67% in the haddock (Reay & Kuchel, 1936) and up to 71% in *Torpedo* (Bailey, 1939). These figures make it very unlikely that all the myosin could be accommodated in the *A* band. The matter appears to have been finally settled by the electron microscope work of Hall *et al.* (1946). By means of a phosphotungstic acid staining technique they demonstrated that the myofibrils are composed of longitudinal filaments (presumably myosin), from 50 to 250A. in width. These filaments are relatively straight and extend continuously through both *A* and *I* bands. Thus the theory that myosin is confined to the *A* bands is no longer tenable.

The A *and* I *bands are both composed of myosin, but in different states of orientation.* An interesting theory based on this hypothesis was put forward by Bernal (1937). He pointed out that van Iterson (1934) had shown that when certain substances which exhibit anisotropy of flow are agitated in a capillary tube the molecules tend to arrange themselves in an alternating right-handed and left-handed spiral pattern. The result is an alternation of birefringent regions, in which the molecules are roughly parallel with the axis of the tube, with non-birefringent regions in which the molecules are less regularly alined. Bernal's attempt to apply these facts to muscle structure and to explain the changes during contraction was extremely stimulating but like so many other theories it has had to be abandoned with the advent of the electron microscope. As we have already seen, the myosin filaments run a relatively straight course throughout the length of the myofibril. There is no gross spiralling either at rest or during strong contraction. It is true that the filaments tend to be rather better orientated in the *A* band than in the *I* band, but it seems very unlikely that the great difference in optical properties between the two bands can be explained by differences of orientation alone.

Szent-Györgyi's theories. During the past eight years a considerable amount of work on the biochemistry of muscular contraction has been performed by Szent-Györgyi and his collaborators (for detailed references see Szent-Györgyi, 1940, 1945*a*, *b*, 1947). Since this work, and the ingenious theories arising from it, have attracted a good deal of attention and have been presented in a most attractive and persuasive manner in a number of lectures and reviews, it is necessary to consider them in some detail.

Until now, when writing of 'myosin' we have referred to the globulin extracted from muscle by slightly alkaline salt solutions (von Muralt & Edsall, 1930; Bailey, 1944). In 1941 Banga & Szent-Györgyi showed that the properties of 'myosin' differ according to the period of extraction. The longer the extraction, the more viscous is the 'myosin'. Straub (1942) then showed that this effect is due to the presence of another protein, 'actin', which is only dissolved after prolonged extraction. 'Myosin' prepared in such a way as to be quite free from actin is found to be water soluble and capable of 'crystallization'. The solution exhibits strong flow birefringence. In order to avoid confusion we shall henceforth refer to this water-soluble myosin as myosin (w-s), following Jakus & Hall (1947). According to

Szent-Györgyi, actin and myosin (w-s) can unite to form a complex called 'actomyosin' which is very viscous in solution and exhibits strong flow birefringence. It thus appears that the classical 'myosin' is really an actomyosin of uncertain composition.

Actin has been shown to exist in two forms. As extracted from muscle, it behaves as a globular protein (*G*-actin). Its viscosity is low and it shows no flow birefringence. As a result of various procedures the globular *G*-actin can be transformed into a fibrous *F*-actin, which is very viscous and shows strong flow birefringence. Jakus & Hall (1947) have studied the *G-F* transformation under the electron microscope. They showed that in alkaline solutions actin is in a corpuscular form. Lowering of the pH brings about a linear aggregation of the particles into long filaments. This process is reversible from a pH as low as 4, the longest filaments being found between pH 6·5 and 7·2. More recently similar results have been reported by Astbury, Perry, Reed & Spark (1947). They studied the *G-F* transformation which occurs under the influence of 0·1 M-KCl and also concluded that the process is one of linear aggregation of corpuscular particles. When myosin (w-s) is added to *F*-actin there is a sudden rise in viscosity. The actomyosin complex shows a birefringence of flow greater than either of its constituents. Actin and myosin (w-s) will combine in all proportions in the presence of magnesium. According to Balenovic & Straub (1942) rabbit muscle normally contains two parts of actin to five of myosin (w-s). The electron microscope again reveals some interesting features. Jakus & Hall (1947) found that myosin (w-s) and classical myosin are quite different, both in general appearance and dimensions. Actomyosin prepared by addition of myosin (w-s) to *F*-actin shows long filaments quite unlike either myosin (w-s) or actin, but resembling classical myosin. Astbury *et al.* (1947) observed a characteristic tendency to form anastomosing networks in actomyosin prepared by mixing eight parts of myosin (w-s) with three parts of *F*-actin. Neither myosin (w-s) nor *F*-actin alone forms such networks. The formation of such a network would account for the high viscosity of actomyosin solutions.

We now come to one of the most remarkable of Szent-Györgyi's discoveries. As was shown by Weber (1934*a*, *b*), if a 'myosin' solution is squirted from a narrow tube into water, a thread is formed. The mechanical properties of such threads have been investigated by Weber (1934*a*, *b*, 1939*a*, *b*) and by Dubuisson (1943). Similar threads can be produced from actomyosin. Szent-Györgyi discovered that such threads could be made to contract by the addition of adenosinetriphosphate (ATP). To understand the significance of this observation it is necessary to know something of the background of recent work on ATP. Since its isolation from muscle by Lohmann (1929) ATP has come to assume a central position in muscle physiology. The chemical evidence points to the breakdown of ATP as one of the earliest events during muscular contraction, and one capable of liberating large amounts of energy (for literature see D. M. Needham, 1937; Kalckar, 1941, 1945; Lipmann, 1941). In 1939 Engelhardt & Ljubimova found that the enzyme adenosinetriphosphatase, responsible for the splitting of ATP, appeared to be identical with myosin itself.

This work was confirmed by Needham (1942) and Bailey (1942). Szent-Györgyi and his pupils also found that both 'crystalline' myosin (w-s) and actomyosin possessed adenosinetriphosphatase activity. More recently evidence has accumulated to show that myosin and adenosinetriphosphatase may not be identical (Singher & Meister, 1945; Polis & Meyerhof, 1946, 1947). Nevertheless, the two are remarkably closely associated, if not identical, so that many of the original arguments are not greatly affected. Following up their first observation, the Russian workers next found that stretched myosin threads showed a reversible increase in extensibility when subjected to the action of ATP (Engelhardt, Ljubimova & Meitera, 1941). They claimed that this effect is only observed if the enzymic activity of the thread is not impaired.

This was the first establishment of a direct link between chemical and mechanical processes in muscle and opened up a new era of research. J. Needham and his co-workers (Dainty *et al.* 1944) showed that very low concentrations of ATP cause a reversible reduction in the flow birefringence and viscosity of myosin solutions. Up to this point the evidence seemed to indicate that the interaction between ATP and myosin is related to *relaxation* rather than *contraction*. At first sight, therefore, Szent-Györgyi's observation that actomyosin threads would *contract* in the presence of ATP was highly important. As Szent-Györgyi himself has said, the demonstration of the existence of protein threads isolated from muscle, which can be made to contract by the addition of another substance present in muscle, is a matter of the greatest philosophical interest. Of that there is no question, but it still remains to be seen whether the phenomenon is of more than philosophical interest—whether in fact the actomyosin thread can be regarded, as Szent-Györgyi claims, as an *in vitro* model of muscular contraction, or whether the contractility of such threads is purely fortuitous.

So far as the basic facts of Szent-Györgyi's work are concerned there appears to be little disagreement. The isolation of myosin (w-s), actin and actomyosin, as well as the demonstration of the contractility of actomyosin threads, have all been confirmed. It is otherwise with the interpretation of these facts, particularly with respect to the theories of muscle structure and muscular contraction which Szent-Györgyi has developed. Let us first of all examine the validity of the actomyosin thread as a model of muscular contraction. It is true that actomyosin threads will contract in the presence of ATP. However, this contraction differs in certain important respects from that of muscle. In the first place it takes place rather slowly, and secondly the fibre shrinks in all its dimensions, unlike a muscle fibre, which becomes shorter and thicker. The 'contraction' is in fact a colloidal synaeresis, or squeezing out of water. Thus, threads containing 98% of water before contraction were found to contain only 50% after contraction. In an attempt to make the conditions in the actomyosin thread approach more nearly to those in muscle, the threads were stretched in order to produce some degree of molecular orientation. According to Szent-Györgyi native actomyosin threads cannot be stretched more than 10–15% without breaking. However, threads partially denatured by zinc sulphate could be stretched by 200%. Such threads would still contract and were now found to become shorter and thicker, like muscle fibres.

Buchthal and his collaborators (Buchthal, Deutsch, Knappeis & Munch-Petersen, 1947) went several steps further. In contradistinction to Szent-Györgyi they found that contractile undenatured actomyosin threads could be stretched to three times their initial length. Moreover, the reaction of the thread to ATP depended on its initial state. If the thread was free from tension, ATP caused it to shorten. If the thread was in a state of tension, however slight, lengthening occurred. It is possible that this effect may explain the discrepancy between the observations of Engelhardt and Szent-Györgyi. It should also be pointed out that Buchthal *et al.* were able to confirm Engelhardt's findings of an increase in extensibility in *non-contractile* threads. Whereas Engelhardt only obtained this effect with enzymatically active threads, Buchthal *et al.* found it with both enzymatically active and inactive threads. Indeed, all the physical changes produced by ATP appear to be quite independent of the enzymatic activity of the actomyosin. Mommaerts (1947) has shown that the fall in viscosity in actomyosin solutions on addition of ATP is also independent of the enzymatic activity.

Thus it must be concluded that, whatever the cause of the mechanical effects produced by ATP, it is unlikely to be related to the enzymic splitting of the latter by myosin. This conclusion is further supported by the work of Mommaerts & Seraidarian (1947), who calculated that whereas in normal muscular activity at least 0·2 mg. of phosphorus is known to be liberated per minute per mg. of myosin, under the conditions present in muscle the enzymic activity of the myosin would only be capable of liberating 0·003 mg. phosphorus per minute per mg. myosin, i.e. the enzymic activity of the myosin could only account for 1·5% of the phosphorus actually liberated. Engelhardt (1946) came to an opposite conclusion, but he does not appear to have allowed for the inhibitory action of magnesium on the adenosine-triphosphatase activity of myosin. It must be admitted that Szent-Györgyi has never regarded the enzymic hydrolysis of ATP myosin as the sole method whereby the former exerts its mechanical effects. The actomyosin thread model does not stand or fall by this criterion alone. A much more serious objection to it is the fact that, as Buchthal *et al.* have shown, the physical changes produced by ATP in actomyosin threads are the reverse of those occurring in muscle. Recently Astbury *et al.* (1947) have shown that the characteristic anastomotic network appearance of actomyosin seen under the electron microscope is lost after addition of ATP.

All the evidence thus points to the action of ATP as bringing about a process of dissolution of elastically active linkages, and as such its role in contraction is uncertain (see also Mommaerts, 1945). For all its shortcomings the actomyosin thread model remains an object of great interest. It is true, as Astbury (1947) points out, that many similar effects can be obtained with artificial fibres containing chain molecules; Mongar & Wassermann (1947) have described interesting contractile threads of calcium alginate for example. Nevertheless, it would be a pity if such a beautiful phenomenon were finally shown to have, as Astbury (1947) believes, 'little or no *special* bearing on muscular contraction'. The actomyosin thread may not be a very good model, but it is difficult to believe that the interaction between myosin and ATP will not one day be shown to be of great importance in muscle physiology. In the opinion of many that day has yet to come.

When we turn to the structural aspects of Szent-Györgyi's work we are confronted with a somewhat confusing profusion of molecular models. In an early paper Szent-Györgyi (1940) attempted to revive the old 'water-shift' theories of muscular contraction (McDougall, 1910; Meigs, 1928; Bernstein, 1905) in a new form. He showed how many of the facts of muscular contraction could be explained by the accumulation of water between a system of relatively rigid fibres with fixed ends. In later reviews (Szent-Györgyi, 1945*a*, *b*) he favoured a 'spiral' theory. According to this the myofibril is composed of a number of long actin threads around which the myosin (w-s) molecules are spirally distributed. Contraction is regarded as due to an unequal shrinking of the actin and myosin (w-s). If the myosin (w-s) loses more water than the actin the turns of the spiral will tend to become closer together, producing a considerable shortening. It followed as a necessary corollary to this theory that in order to ensure close packing the spirals of adjacent threads within the myofibril had to be in alinement. If the periodicity of the spiral is assumed to be that of the *A* and *I* striations, then according to Szent-Györgyi the latter have no real existence, but are merely an optical illusion resulting from the spiral arrangement. As evidence in support of this idea Szent-Györgyi quoted experiments in which muscle fibrils were embedded in gelatine within glass capillary tubes. The latter were placed under the microscope and rotated about their axes. It was claimed that the striations within the fibrils moved continuously, like a corkscrew, while the tube was in rotation.

Now both the conditions and the interpretation of these experiments are open to criticism. In the first place the order of the shifts in the striations which Szent-Györgyi claimed to have observed was very small—about 1 μ for a rotation of 180°. It seems rather improbable that the method of fixing the glass capillaries (paraffin wax) was sufficiently rigid and free from slight shifts. I have attempted to verify these experiments in another way, using the most delicate (de Fonbrune) micromanipulator available, but even with this instrument the fibril could not be kept absolutely still (unpublished observations). The photographs shown by Szent-Györgyi (1945*b*, p. 102) can be interpreted in another manner. Anyone who has observed teased isolated muscle fibres is familiar with the fact that the striations or disks do not always appear to lie exactly perpendicular to the axis of the fibre. If the disks were slightly out of the perpendicular then they would appear to move as the fibre was rotated. Yet another explanation was offered by Szent-Györgyi's collaborators, Matoltsy & Gerendás (1947). It appears that in Szent-Györgyi's original experiments the microscope focus was altered during the rotation of the tube. If the focus was kept constant no continuous shift was observed. We shall discuss the evidence for the real existence of the cross-striations in a later section. At this stage we need only state that the electron microscope has dealt the death-blow to all theories in which the striations are regarded as an optical artefact. Szent-Györgyi (1947) has now withdrawn his spiral theory in its original form since this is incompatible with the electron microscope findings of Hall *et al.* (1946). If any spiralling exists it must be on a molecular scale far smaller than has hitherto been revealed by the electron microscope.

There is one other aspect of Szent-Györgyi's work which it is necessary to mention, even though it perhaps concerns function more than structure. In his recent book (1947) contraction and relaxation are regarded as two distinct states with no intermediate condition. An actomyosin micelle is either fully relaxed or fully contracted. This is by no means an unlikely supposition, but the conclusions drawn from it by Szent-Györgyi and his school are somewhat strange. In particular the attempt to derive thermodynamic data from mechanical experiments on muscle slices has led to some curious results. In these experiments (Szent-Györgyi, 1947, p. 45): 'L. Varga considered the muscle fibre to be a similar perfect actomyosin thread and used as experimental material the psoas of the rabbit, which is built of parallel-running fibres. He washed out the muscle *in situ* with ice water and cut it into slices on the freezing-microtome in such a way that each slice contained one sheet of parallel running fibres. After the length was measured, the slice was transferred into a KCl-ATP solution of known temperature and its length measured again after maximal contraction was reached.' An equilibrium constant was then calculated based on the assumption that if the muscle is contracted to $x\%$ of the maximum, $x\%$ of the actomyosin micelles are fully contracted, the rest being fully relaxed. Szent-Györgyi resumes: 'The natural logarithm of the equilibrium constants plotted against $1/T$ gave a straight line, which proved both of Varga's suppositions to be correct: that muscle actually behaves as an ideal thread, that contraction and relaxation are distinct states; and that an actomyosin micel is either fully relaxed or fully contracted, there being no transitional states.'

Leaving aside the question of whether or not this is an example of argument in a circle, one may legitimately ask whether the type of material used by Varga bears any relation whatsoever to normal muscle? I have yet to find a histologist who claims to be able to cut 'one sheet of parallel-running fibres' on a freezing-microtome. Unless Varga used some very special method, it is clear that his material must have consisted partly of relatively intact fibres and partly of sliced and damaged fibres. After washing in ice water, freezing, sectioning and thawing, the material might consist of a mixture of dead fibres and fibres in various states of viability. One would hardly expect to derive much quantitative thermodynamic data from such material. However, by plotting the logarithm of the equilibrium constant against the reciprocal of the absolute temperature, Varga claims to be able to deduce the free energy charge and heat of reaction of the contractile process. He finds that in rabbit's muscle there is a fall of free energy of 7000 cal. at 37° C. The heat of reaction is −56,000 cal. so that muscular contraction is an endothermic process! At 0° there was no drop of free energy so that rabbit muscle cannot contract at 0°. Szent-Györgyi (1947) resumes: 'It was rather shocking to find a frog swimming about in ice water after the conclusion of these experiments, and we had to decide whether the frog or Mr Varga was wrong.' New experiments showed that the recalcitrant frog was able to swim by virtue of the fact that frog's muscle still shows a small drop in free energy at 0°, the drop becoming zero at $-3°$.

The most remarkable deduction made from these experiments is that contraction is an endothermic process, whereas relaxation can only occur if there is an increase of free energy to the extent of 7000 cal. (at 37°). Now it has frequently been suggested that relaxation is an active process (see Ramsay, 1944; Ramsay & Street, 1940), but this appears to be the first time that anyone has suggested that contraction is endothermic. This idea contradicts the entire work of A. V. Hill over the last 30 years. Hill has repeated his experiments with greatly improved technique (Hill, 1938) and has shown that even the most elementary muscular contraction is highly exothermic. There is no evolution of heat directly associated with relaxation. Hill has also pointed out that Szent-Györgyi's deduction that the force of contraction of frog's muscle at 0° should be very small is not borne out in practice. Szent-Györgyi attempts to resolve the difficulty by suggesting that Hill and Varga are measuring the heat changes of two quite different processes. One can only express complete agreement with this statement.

It has only been possible to discuss certain aspects of Szent-Györgyi's work, and those with undue brevity. For more details Szent-Györgyi's papers should be consulted. (See Bailey, 1946, 1947, for a review of Szent-Györgyi's biochemical work.) It would be wrong to pretend that his ideas are generally accepted by workers on muscle structure and physiology. Nevertheless, although certain of his conclusions are open to severe criticism, his investigations have been most stimulating and have opened up several fruitful avenues. In particular they have already led to a better understanding of the muscle proteins, and it seems impossible to doubt that they will ultimately lead to a fuller knowledge of muscle structure and function.

Carey's theory. A novel attempt to explain the appearance of the cross-striations has been made by Carey (1936, 1940, 1942). According to his theory the striations are the result of a system of standing pressure waves due to mechanical vibrations within the muscle protoplasm. The motor end-plate is regarded as the centre of a vibration which is propagated through the liquid protoplasm to the ends of the fibre, whence it is reflected back to the centre, forming a system of standing waves. Carey has demonstrated how such waves can be produced in models and in a series of beautifully illustrated papers has attempted to show that the motor end-plate is capable of undergoing amoeboid movement. Unfortunately the underlying physical concepts, as stated by Carey, are somewhat difficult to grasp. Thus in one paper (Carey, 1936, p. 264) it is stated: 'There is a relation between velocity, wave lengths and frequency or number of vibrations per second. By knowing the velocity (v) of the nerve impulse and the wave length (l) measured as one cross striation of a muscle fibre, the high frequency (n) of motion of the motor nerve ending and the liquid molecules of the muscle fibre may be found in the formula $v = lxn$.'

It is difficult to see why the velocity of the electrical nerve impulse should govern the wave length of the mechanical pressure waves, but assuming the correctness of the formula and taking the velocity of the nerve impulse in mammalian medullated nerves at 37° C. as 100 m./sec., and the distance between cross-striations as 2μ we find $n = 50 \times 10^6$ vibrations per sec. This is an enormous frequency of vibration, and it is hard to see what structures in muscle can be capable of such rapidity of movement. Certainly one would think that if the motor end-plate moved at such a rate it would generate so much heat as to destroy itself.

As regards Carey's evidence for amoeboid motion of the motor end-plates, so far as I am aware all this work has been done on fixed and stained material. Carey (1942) has certainly shown that the end-plates tend to undergo characteristic changes in morphology under certain conditions, but this is hardly evidence of 'amoeboid' activity. In the course of prolonged observations on the living motor nerve endings in insect larvae under phase-contrast microscopy I have not observed any special activity of the end-plate. The latter tends to move passively during muscular contraction, but no amoeboid movement can be seen.

As further evidence that 'the so-called sarcomere is a morphologic myth', Carey, Zeit & Massopust (1942) claim to have observed an increase in the number of striations with temperature in the living muscles of the freshwater shrimp. This conclusion has been criticized by Jordan & Speidel (1942), on the grounds that Carey *et al.* counted some sarcomeres twice. I have been unable to confirm Carey's results on living insect larvae.

There are numerous points which make it difficult to accept Carey's theory of the striations. If the motor nerve-ending played the important role ascribed to it, one would expect very considerable changes in the striations in denervated muscle, and especially in isolated fibres cut in two parts, one of them containing no nerve-ending. Such pieces of muscle fibre retain their striations and remain contractile for several hours in the complete absence of a nerve-ending.

The cross-striations are due to structures lying outside the myofibrils. The main protagonists of this theory have been Ettisch (1933) and Liang (1936). According to the former, when muscle fibres are observed under dark-ground illumination in such a way that the light strikes the fibre in a direction parallel to its axis, only the cross-striations can be seen. If the light strikes the fibre in a direction perpendicular to the axis the cross-striations become invisible but the longitudinal fibrillar structure is clearly seen. These results are not altogether surprising, but it is difficult to see why they should be interpreted as showing that the myofibrils themselves are not striated. The great difficulties in interpreting obscure optical effects in a structure such as striated muscle in which scattering, interference, and diffraction must play a large and confusing part do not allow us to accept such a view.

Liang (1936) has produced other evidence based on observations on the effects of certain contracture-producing drugs on the structure of freshly teased muscle fibres. Such fibres are said to lose their cross-striation under the influence of chloroform, caffeine and quinine, and the myofibrils to become uniformly birefringent. Liang believes that the muscle fibre is composed of uniformly birefringent myofibrils, around which there is a network of material which aggregates at periodic intervals, giving the fibre a striated appearance. It is not clear why contracture-producing drugs should attack this network alone. If the action of caffeine is observed under the microscope, the entire muscle substance seems to be affected. The myofibrils contract and the striations tend to become drawn together, in some cases forming contraction bands. I have been unable to confirm the loss of cross-striations in a muscle undergoing a caffeine contracture, except in so far as contraction bands or clots are formed. On the contrary, the cross-striations appear to be very stable structures and there is usually some remnant of them to be seen even in fibres undergoing advanced disintegration. Nor has it been possible to corroborate Liang's statement that acids disrupt muscle fibres too much to allow observations of structure. It has been shown (Barer, 1947*a*) that acid-treated fibres may retain both their cross-striation and their myofibrillar structure, and it has been possible to pull out striated myofibrils from such fibres.

Other evidence against the existence of a perforated network within the sarcoplasm will be considered later. For the moment we may conclude the discussion by remarking that the electron microscope has afforded no support for this theory. There does appear to be a definite cross-striation within the myofibril.

The different properties of the A *and* I *disks are due to the presence of different substances.* At first sight this seems to be identical with the first theory discussed above. There is a subtle difference however. Whereas the first theory postulated that the myosin or actomyosin is confined to the *A* band, it is hoped to suggest in the present theory that while both *A* and *I* disks are composed fundamentally of the same contractile protein, the presence of *other* substances confined to one or other band may affect the physical properties of the protein within those bands. I have already put forward such a theory tentatively (Barer, 1947*a*) and somewhat similar ideas have been expressed recently by others (Dempsey *et al.* 1946; Matoltsy & Gerendás, 1947). We must first of all examine the evidence in favour of the periodic distribution of different chemical substances within the *A* and *I* bands. Such evidence is based chiefly on histochemical methods, micro-incineration and ultra-violet microscopy.

As we have already seen, the electron microscope studies of Hall *et al.* (1946) have shown that both *A* and *I* bands contain longitudinal protein filaments. In order to explain the fact that the *A* bands appear darker than the *I* bands in the electron microscope (i.e. their electron scattering power is greater) Hall *et al.* suggested that there is a high concentration of salts in the *A* band. As early as 1905 Macallum had claimed that potassium is confined to the *A* bands. Taken by itself his work cannot be regarded as conclusive, since it was done at a time when no special attention was paid to such point as specificity of reaction or efficiency of fixation (see Danielli, 1946). Hall *et al.* point out, however, that the *A* band appears to have a special affinity for phosphotungstic acid, which is known to form an insoluble complex with potassium (Rieben & Van Slyke, 1944).

It might be expected that some confirmatory evidence regarding the distribution of potassium would be obtained from micro-incineration studies, but unfortunately the results are not altogether clear cut. The early work of Scott (1932, 1933) and others showed that the ash residue obtained after incineration of sections of fixed striated muscle at about 600° C. was arranged in striations corresponding to the *A* bands. The *I* bands appeared to be free from ash. Similar results have been obtained by the majority of workers using this technique. Thus, Kruszyński (1938) found that the ash residue was almost entirely confined to the *A* bands of the myofibrils, the sarcoplasm and *I* bands being relatively free from ash. He also claimed that the *M* band, when seen, was free from ash. Loreti (1941) arrived at similar conclusions from a comprehensive study of insect muscles, though he claimed that the sarcoplasm contained a little ash. One of the chief difficulties of the micro-incineration technique is that unless special steps are taken to prevent diffusion of salts from one part of the cell to another, the results are apt to be misleading. These difficulties do not always appear to have been appreciated by users of the method. However, Scott & Packer (1939*b*) have attempted to avoid such fallacies by the use of freeze-drying instead of ordinary methods of fixation, and the avoidance of contact of the specimen with water. Following micro-incineration of such material, a heavy white ash was found in the *A* bands, and a light bluish ash in the *I* bands. The nature of the ash distribution in relaxed muscle is uncertain, but by heating the specimen on the cathode of an electron microscope Scott & Packer (1939*b*) claim to have shown that calcium and magnesium occur in high concentration in contraction nodes. Other workers have also observed small quantities of ash in the *I* bands under some circumstances, particularly when the temperature of incineration was rather low (400° C.). Kruszyński (1938) suggested that potassium is present in the *I* bands but tends to volatilize at high temperatures. It thus appears that most workers on micro-incineration are agreed that most of the ash is localized in the *A* bands. The nature of the ash is uncertain, but it seems more likely to be calcium and magnesium than potassium. There is some evidence that the latter may occur in the *I* bands. It will be noticed that this is the reverse of what Hall *et al.* have suggested, but as we shall see below the matter is by no means finally settled.

Ultra-violet microscopy provides further evidence for the existence of different substances within the *A* and *I* bands. Caspersson & Thorell (1942) have shown that living muscle fibres photographed in ultra-violet light of wave-lengths between 2400 A. and 2950 A., with a wide condenser aperture exhibit a cross-striated appearance. The conditions of the experiment are such as to leave no reasonable doubt that this cross-striation is mainly due to a true absorption of ultra-violet light. The interesting feature about these results is that according to Caspersson & Thorell the strongly absorbing bands are identical with the *I* bands. This conclusion was based on indirect comparisons between fibres photographed in polarized light and in ultra-violet light. Hoagland (1946) has recently confirmed this result by making a direct comparison on the same fibre, one-half of which was photographed in polarized light, the other half in ultra-violet light. In view of this it appears reasonably certain that the *I* band contains a substance showing strong absorption in the 2400–2950 A. region. Now according to Caspersson & Thorell the only substances present in muscle which show strong absorption in this region are the proteins and the purine derivatives, of which adenylic acid and ATP constitute over 95%. By plotting absorption curves for the *A* and *I* bands and comparing them with the known curves for adenylic acids and myosin the Scandinavian workers conclude that the *I* bands of resting fibres are very rich in adenylic acids, which are mainly responsible for the strong ultra-violet absorption. In muscles photographed after undergoing a number of contractions the strongly absorbing substance appears to diffuse into the *A* bands.

Now, if Caspersson & Thorell are correct in assuming that the adenosine compounds are mainly confined to the *I* bands at rest, one may speculate as to what effect this might have on the birefringence of the myosin within those bands. Dainty *et al.* (1944) showed that ATP reduces the flow birefringence of myosin solutions. It is not unlikely that it may produce a similar effect in the myosin filaments in the *I* bands. However, the criteria used by Caspersson & Thorell for identifying the strongly absorbing substance with adenosine compounds are not as definite as one would wish. In the present state of our knowledge it is somewhat dangerous to assume that adenosine compounds and myosin are the only substances in muscle which absorb strongly in the 2400–2950 A. region. As Szent-Györgyi and his school have shown, muscle contains a large number of proteins, and their distribution within the striations is largely unknown. Matoltsy & Gerendás (1947) have recently claimed that a phosphorus-containing protein can be isolated from the *I* bands. This would presumably exert some effect on ultra-violet absorption. We may also note in passing that according to Szent-Györgyi's views there can be no free ATP in muscle. All the ATP should be intimately bound to the actomyosin molecule. The work of Caspersson & Thorell is thus extremely suggestive in that it provides further evidence for differences of distribution of chemical compounds within the striations; even if the ultra-violet absorbing substance turns out not to be composed of adenosine compounds it is nevertheless of considerable interest and importance.

An interesting extension of Caspersson & Thorell's work, which has led to somewhat unexpected results, has been made by Engström (1944). As he has pointed out, since the adenylic acids contain large amounts of phosphoric acid it might be possible to locate the former by micro-incineration methods. Engström first confirmed the statement that the *I* segments show strong absorption in the ultra-violet. He then took photographs of muscle fibres in the ultra-violet and compared them with photographs of the ash residue obtained after micro-incineration of the same fibre. According to his results, the ash is almost entirely located in the *I* segment. This is in complete contradiction to the statements of almost every other worker who has performed micro-incineration work on muscle fibres. It is at present impossible to make any definite statement concerning the problem; clearly these conflicting results call for a reinvestigation of the whole question. It is true that Engström appears to *define* the *I* band as the ultra-violet-absorbing band in most of his material, but he also claims to have checked this in some cases with the polarizing microscope. It must be admitted that the quality of the photographs shown in his paper are not such as to inspire confidence, but this may be due to poor reproduction. Most of his work has involved the use of fixatives, but he appears to have used frozen-dried material in some cases.

We must, therefore, regard the problem as in abeyance, but although we cannot say with certainty that any particular substance is located in any particular band we can say quite confidently that different substances are located in different bands. Further evidence in favour of this view comes from ordinary histological and histochemical methods. The mere fact that the *A* and *I* bands react differently to various dyes suggests that they may contain different substances. An important investigation of the chemical cytology of striated muscle has been carried out by Dempsey *et al.* (1946). They showed that the *A* and *I* bands behave differently to acid and basic dyes at different hydrogen ion concentration. Thus at pH 5·5–7·0 only the *A* band and the *Z* line stain with methylene-blue, the *I* band remaining unstained. On the other hand, the *I* band stains strongly with Orange *G* at pH 4–6, whereas the *A* band is poorly stained. The nature of the basophil substance in the *A* band is unknown. Dempsey *et al.* point out that it behaves towards dyes quite differently from the basophil substance in Nissl granules, cartilage, mucus and most cells. The basophil substance was not affected by digestion of the specimen with ribonuclease. I, too, have been unable to observe any alteration in the striations of muscle fibres after digestion with either ribonuclease or desoxyribonuclease, following the technique used by Sanders (1946). Another indication of chemical differences between the two bands is provided by the fact that the *I* bands are argyrophil, particularly after impregnation by the Bodian protargol method.

Perhaps the most interesting results of Dempsey *et al.* are those concerning the distribution of lipoids in the muscle fibre. The *I* bands were found to stain heavily both with Sudan black and with the Smith-Dietrich method for phospholipins (see Baker, 1944). After thorough extraction of the sections by 3 hr. treatment with boiling alcohol, the affinity for Sudan black practically disappeared, and it was then found that the *I* bands showed a great increase in birefringence, approaching that of the *A* bands. Dempsey *et al.* suggest that since myosin filaments show positive birefringence, whereas lipoids usually show negative birefringence, it may be possible for the birefringence of the myosin in the *I* bands to be masked by the presence of lipoids in those bands.

Another claim to have found a negatively birefringent substance in the *I* bands has been made by Matoltsy & Gerendás (1947). They found that after extraction of myosin and actin from pieces of muscle, the *A* bands became non-birefringent whereas the *I* bands showed a strong negative birefringence, equal to the original positive birefringence of the *A* bands. The negative birefringence was believed to be due to a phosphorus-containing protein which could be extracted by a urea-salt solution. It was further suggested that this protein is present in the *N* band and is responsible for neutralizing the positive birefringence of the myosin.

Thus we see that the general trend of opinion is that while both the *A* and *I* bands are fundamentally composed of the same material (myosin or actomyosin), the presence of minor constituents in one or other band may be responsible for differences in physical properties. It is perhaps too early to decide in favour of any particular explanation of the isotropy of the *I* band. The claim of Caspersson & Thorell (1942) that ATP is confined to the *I* band requires further investigation. Both Dempsey *et al.* and Matoltsy & Gerendás have used somewhat drastic procedures in the course of their work and it is scarcely possible to decide whether a negatively birefringent lipoid or protein (or perhaps both) is responsible for the masking action. The basically important fact that emerges from all this work is that different substances are present in the two bands and that these may very well account for differences in optical properties. How far they may be responsible for differences in mechanical properties is a matter for conjecture, but if, for example, most of the salts were localized in one band, it is difficult to imagine that the colloidal and mechanical state of the myosin or actomyosin in that band would not be profoundly different from that of the same protein in the salt-free band. Similarly the presence of ATP in one band and not the other would be expected to lead to equally profound differences.

VII. THE ORGANIZATION OF THE MUSCLE FIBRE

We must now consider how the various components of the muscle fibre—sarcolemma, sarcoplasm and myofibrils—are interrelated and integrated to form a single contractile unit.

How is the sarcolemma related to the muscle contents? This question raises several important problems. Whatever the actual nature of the sarcolemma—whether it is to be regarded as a plasma membrane or as a mechanical sheath (or both)—it is clear that the contractile force has to be transmitted via the sarcolemma to the surrounding connective tissue. At the ends of each muscle fibre communication is somehow established with tendon fibrils. The nature of this communication has been the subject of considerable debate. There are two main schools of thought: those who believe that the myofibrils are directly continuous with the tendon fibrils (Schmidt, 1927; Carr, 1931; Butcher, 1933), and those who believe that there is no direct continuity, the myofibrils being separated from the tendon and connective tissue fibrils by the sarcolemma (Baldwin, 1913; Goss, 1944; Long, 1947). An intermediate position was adopted by Schultze (1912), who regarded the sarcolemma covering the ends of the fibre as a perforated sieve-like structure, through the holes of which connective tissue fibrils passed to establish contact with the myofibrils.

There seems little prospect of settling this controversy by the conventional use of fixed and stained sections. The problem is essentially one involving three dimensions and can only be solved by observations of whole muscle fibres. Observations on living muscle fibres and microdissection studies offer no support for the idea that there is any special connexion between myofibrils and tendon fibrils. Retraction clots often occur in the region of the muscle tendon junction, leaving a clear area of sarcolemma between the tendon fibrils and the clot. Spontaneous herniation of the muscle substance has been seen to occur close to the muscle tendon junction following treatment with dilute acids (Barer, 1947*a*), and again a clear area of sarcolemma is left. Microdissection of such material suggests that the sarcolemma is continuous over the ends of the fibre, the tendon fibrils being attached to the outside of the sheath. Nor does there appear to be any specially intimate connexion between the ends of the myofibrils and the surrounding sarcolemma.

In fact, there is very little evidence of any structural continuity between any part of the muscle substance and the sarcolemma. It is frequently stated that the *Z* bands are attached to the sarcolemma (Häggquist, 1931). If this is so, the attachment must be very loose and readily broken down. We have already seen that the sarcolemma can be made to separate from the muscle substance in a large number of ways, using mechanical, chemical, pharmacological, thermal or electrical methods. The very non-specificity of these methods suggests that the attachment if any, is very weak.

How then is the force of contraction transmitted to the tendons and connective tissue? We may well imagine the relationship between the sarcolemma and the underlying muscle substance as a frictional or viscous one. Many of the mechanical properties of the muscle fibre can be shown in models such as that proposed by Gasser & Hill (1924) composed of a thin rubber tube containing a viscous fluid or gel. A similar model has been proposed recently by Schoepfle & Gilson (1945) in order to explain the latency relaxation observed by Sandow (1944). It is not difficult to imagine that contraction of a viscous gel would exert a pull on a surrounding elastic membrane and thence on to adjacent connective tissue fibrils. The formation of retraction clots and the readiness with which the muscle substance can be made to retract from the sarcolemma is easily explained on this basis.

The Z disk. The *Z* band has played an important part in many theories of muscle structure. It is sometimes referred to as 'Krause's membrane' and has been regarded as the terminal membrane of the contractile unit, the sarcomere. From time to time it has been claimed that the *Z* bands of adjacent myofibrils are continuous with one another through the intervening sarcoplasm, and various authors speak of the *Z* 'membrane' which stretches right across the fibre, linking up with myofibrils to be attached peripherally to the sarcolemma. Häggquist (1931), for example, regards the *Z* membrane as collagenous and structurally continuous with the sarcolemma. We have already seen that the latter claim is based on very slender evidence; the supposition that the *Z* band is collagenous is rendered highly improbable by the electron microscope. Collagen has a characteristic striated structure when seen under the electron microscope, and this is absent from the *Z* bands.

The idea that any permanent transverse membranous structures exist within the muscle fibre as a whole is rendered rather dubious by several observations. The freezing experiments of Chambers & Hale (1932), already referred to, show that columns of ice can advance unhindered between the bundles of myofibrils. Speidel (1939) has observed muscle nuclei moving between the myofibrils without any apparent hindrance from cross-membranes. The same author quotes Chambers and Sichel as stating that oil droplets may be pushed along freely inside the fibre. I have obtained similar results by micro-injection of dyes.

Finally, the fact that myofibrils or groups of myofibrils can contract independently or asynchronously would be very difficult to explain if adjacent myofibrils were structurally connected by cross-membranes. We must agree with Speidel (1939) that: 'Until its nature is more definitely determined, perhaps the non-committal term "*Z* disk" is preferable to the term "*Z* membrane".' The evidence against the existence of a *Z* membrane applies equally well against theories which ascribe the existence of other striations to a perforated network surrounding the myofibrils. Such theories (Ettisch, 1933; Liang, 1936) have already been discussed above.

Spiral theories. From time to time the statement is made that the striations of the muscle fibre are not to be regarded as disks but as optical sections of a spiral or helicoid structure. Such theories have been championed by Tiegs (1928, 1932, 1934), D'Ancona (1930), and Aurell & Wohlfart (1936). It must be admitted that many of the arguments and illustrations which have been advanced in favour of such views are extremely difficult to refute. We have already seen that the electron microscope enables us to exclude the existence of a spiral structure within the myofibril itself. The theories at present under discussion refer to a spiral organization on a different scale, namely within the muscle fibre as a whole. This implies that the myofibrils are related to each other in such a way as to cause corresponding striations to form a spiral. There is little agreement, even among the supporters of the spiral theories, upon the actual nature of the spiral. Thus, Tiegs (1934) believes that the common arrangement is a double spiral. D'Ancona, on the other hand, states that a single spiral is more usual, while Aurell & Wohlfart deny the existence of multiple spirals. Some of these theories presume the existence of a continuous spiral *Z* membrane, and Tiegs (1934) has even suggested that this membrane, which he believes to be innervated by fine filaments from the motor nerves, is responsible for conduction of the excitatory process. As we have seen, it is unlikely that any continuous transverse membrane, spiral or otherwise, can exist in the muscle fibre.

Perhaps the main argument against the spiral theory is that practically all the special appearances it claims to explain can be equally well accounted for on a simpler basis. Feneis (1938), for example, has shown that Aurell & Wohlfart's (1936) diagrams of 'striation wedges', 'vernier effects', and spirals may well be due to a lack of perfect alinement between adjacent groups of myofibrils. In support of this Speidel (1939) has pointed out that 'vernier effects' can be produced experimentally in mild injury of a living fibre. This causes slight contraction of irritated groups of myofibrils leading to a lack of proper alinement. Anyone who has studied living fibres will have seen such effects and it is very probable that a similar phenomenon occurs in many fibres during the process of fixation. Speidel's observations on myogenesis also afford no support for the spiral theory.

Another objection to the theory is that under certain circumstances muscle fibres can be made to undergo cleavage into transverse disks. This was in fact pointed out by Bowman (1840), by whose name the disks are known. Such discoid disintegration has been observed after treatment with acids, alcohol and after formalin fixation (Clark, 1946). It is true, as Tiegs has argued, that these disks may in fact be fragments of a spiral. One can only state that this does not seem to be the case when acid-treated fibres are split into disks by microdissection under the microscope. Nor is there any evidence of uncoiling of a spiral when the swollen muscle substance is allowed to flow through a spontaneous rupture of the sarcolemma (Barer, 1947*a*). Certainly teased insect fibres occasionally appear to have a definite spiral structure. I am loath to accept this interpretation because on a few occasions I have found that when such a 'spiral' fibre is seized between microneedles and gently stretched the spiral is lost, to be replaced by an equally definite discoid appearance. This requires further investigation, but for the moment we may stress once again the need for caution in interpreting the optical image of so complex a structure as the muscle fibre.

In conclusion, we may mention the work of Buchthal & Knappeis (1940) on the diffraction spectra produced by living muscle fibres. Such work (see also Nicolai, 1936; Sandow, 1936) has shown that striated muscle behaves as a very regular diffraction grating of simple pattern with a periodicity corresponding to the distance between striations. The complicated diffraction spectra which would be expected from a spiral structure have never been observed.

To sum up, the balance of evidence is against the view that a spiral organization exists within the muscle fibre. It is perhaps possible that such a structure may occasionally occur, particularly in certain insect muscles, but further investigation of the problem is necessary.

The alinement of the striations. We have already seen that there is good evidence that the muscle fibre contains a large number of striated myofibrils. At the same time we have referred to the fact that these myofibrils usually appear to be very accurately alined, so that the various bands of any myofibril are related to the corresponding bands of its immediate neighbours. We have also seen that certain treatments may cause the fibre to break up into a series of transverse disks. How are we to explain this accurate alinement of longitudinal fibrils? The simplest and orthodox explanation is that the myofibrils are interconnected laterally by transverse membranes. We have discussed the evidence for this view above and have seen that many facts are difficult to explain on this basis. The alternative suggestion is that the myofibrils are kept in alinement by some form of intermolecular force (Barer, 1947*a*). As regards the nature of such a force little can be said. What we may stress, however, is the probability that the forces in the region of the various bands are likely to differ quantitatively, and perhaps qualitatively as well. As already stated, there is probably a periodic distribution of different substances in the different bands of the myofibril. This, in turn, may lead to differences of physical properties of the myosin within those bands. Differences in salt concentration must almost certainly lead to profound differences in ionic atmosphere and electrical charges along the fibre (for a discussion of electrostatic potentials in single muscle fibres see Buchthal & Peterfi, 1934) and these must in turn affect the nature and strength of the intermolecular forces. At least three possibilities suggest themselves.

(1) *Surface forces.* We can readily imagine that the surface attraction between two *A* bands or two *I* bands is greater than between an *A* and an *I* band. This would tend to produce alinement of striations where the myofibrils are in contact. Since such surface forces are generally only operative over very short distances it might be objected that they would be ineffective where the myofibrils are separated by an appreciable thickness of sarcoplasm. This is quite true, but it should be pointed out that even if the adjacent myofibrils are in actual contact for only a small fraction of their lengths they would be automatically alined, assuming that the distance between striations is the same in all myofibrils.

(2) *Long-range colloidal forces.* There are numerous indications that long-range forces, probably electrical in nature, may exist between colloidal particles. Thus Bernal & Fankuchen (1941) found evidence of forces in tobacco mosaic virus solutions operating over distances of at least 450 A. Equilibrium between plate-like particles can be maintained over distances up to 8000 A. (0·8μ) (Bergmann, Low-Beer & Zocher, 1938). The origin of such forces is obscure, but Levine (1939) has developed a theory which seeks to explain them on the basis of interpenetration of ionic atmospheres (see also Levine, 1946; Verwey & Overbeek, 1946). It is tempting to believe such forces may be of great biological importance. Danielli (1945) has suggested that they may explain the shape of the red cell and rouleaux formation. According to Rothen (1945, 1946, 1947) antigen-antibody reactions can occur even when the reacting substances are separated by films of barium stearate. Enzymes have been found to act on their substrates when separated by films over 100 A. in thickness. He suggests that such interactions may occur across thin biological membranes.

The assumption that long-range colloidal forces, acting through thin layers of sarcoplasm, may be responsible for the alinement of myofibrils is perhaps attractive, but nevertheless entirely speculative. We have little information regarding the thickness of sarcoplasm between the myofibrils. Buchthal & Knappeis (1940) suggested, on the basis of their diffraction spectra measurements, that a layer of sarcoplasm 5μ thick intervenes between bundles of myofibrils. This distance is rather great, even for the type of force under discussion, but the same argument can be applied here as in the case of surface forces: the adjacent myofibrils or bundles of myofibrils need only approach each other closely at one or two points for alinement to be established along their entire lengths. Buchthal & Knappeis (1940) have also pointed out that not only are the myofibrils within a given fibre in alinement, but there is often an excellent alinement of the striations in adjacent muscle fibres. It is possible that the forces which operate between adjacent myofibrils in the same fibre may also operate between the myofibrils in two different but contiguous muscle fibres.

(3) *Hydrogen bonds.* It is well known that hydrogen can form links between certain atoms, particularly nitrogen and oxygen (see Pauling, 1940; Hunter, 1946). Such bonds have been shown to exist in proteins (Buswell, Krebs & Rodebush, 1940; Ellis & Bath, 1938) and may be of biological interest (Astbury, 1940). The possibility arises that hydrogen bonds may occur between the proteins in the myofibrils and those in the surrounding sarcoplasm. The number and strength of such bonds may depend on local conditions, i.e. the presence of salts and other substances.

While it is not difficult to postulate various types of forces which may act between the myofibrils it is scarcely possible to present any direct evidence for their existence. The reason for the alinement of cross-striations remains a mystery, the solution of which may depend on a better understanding of the nature of colloidal forces. Even if we accept the thesis that the local properties of the myofibril are influenced by a periodic distribution of different substances, we are still no nearer an explanation as to why this periodic distribution should occur. Possibly some process similar to the formation of Liesegang rings is involved. A study of the colloid chemistry of the myoblast at the earliest stage of development of cross-striation might shed some light on the problem.

VIII. SUMMARY

1. The muscle fibre is regarded as a bundle of myofibrils, embedded in a viscous matrix, the sarcoplasm, the whole being surrounded by a delicate sheath, the sarcolemma.

2. The sarcolemma is a thin, apparently structureless membrane. Preliminary study with the electron microscope suggests that it may be less than $0{\cdot}1\,\mu$ thick. No fibrils can be seen with the electron microscope, but a large number of small nodules lie scattered over the membrane. The sarcolemma is probably not collagenous in nature.

3. The sarcoplasm is a viscous, protein-containing gel which surrounds the myofibrils. Mitochondria, glycogen and fat droplets are scattered throughout the sarcoplasm. Several enzymes have been isolated from what is believed to be the sarcoplasm. This suggests that the latter may be the site of important metabolic activity. The question of sarcoplasmic contractility is not yet settled. There is good evidence that it may occur in certain cells (myoblasts, striated fibres of tadpoles and insect larvae).

4. The evidence in favour of the existence of myofibrils as contractile units is discussed. The fact that individual myofibrils or groups of myofibrils can be made to contract independently, and sometimes asynchronously, is strong support for this view.

5. The electron microscope shows that the myofibrils consist of relatively straight protein chains. Several different striations (*A*, *I*, *Z*, *M*, *N*, *H*) can be recognized. There is no gross difference in orientation between the protein chains in the various bands, nor is there any evidence of a spiral structure.

6. Several aspects of Szent-Györgyi's work are discussed. While this work has led to a better understanding of the muscle proteins, the theories of muscle structure arising from it have found no general acceptance.

7. Other theories attempting to account for the existence of the cross-striations are reviewed. It is suggested that there is a periodic distribution of different chemical substances in the different bands. This may affect the physical, mechanical, and chemical

properties of the myosin in those bands, and may account for the differences in birefringence between the *A* and *I* bands.

8. Evidence is presented against the existence of a *Z* 'membrane' or other transverse membranes uniting the myofibrils. No structural connexion is believed to exist between the sarcolemma and the underlying sarcoplasm and myofibrils. The relationship may be a purely viscous or frictional one.

9. Reasons are suggested for the perfect alinement of the striations in neighbouring myofibrils. A full explanation may only be possible as a result of a better understanding of intermolecular and colloidal forces.

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LE CONTRÔLE HORMONAL DE LA DIFFÉRENCIATION DU SEXE

PAR A. JOST
Laboratoire de Physiologie générale
Muséum National d'Histoire Naturelle, Paris

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I. INTRODUCTION

L'hypothèse selon laquelle les sécrétions des gonades embryonnaires jouent un rôle directeur dans la différenciation du sexe a été émise pour la première fois par Bouin & Ancel en 1903, dans une note 'Sur la signification de la glande interstitielle du testicule embryonnaire'. Ces deux savants qui venaient de montrer la part des cellules interstitielles dans l'élaboration de l'hormone mâle chez l'adulte, constatèrent que dans l'embryon de Porc très jeune, vers le stade de 3 cm., le testicule renferme des cellules interstitielles volumineuses dont l'aspect histologique rend l'activité sécrétrice très probable. Ils supposèrent que la sécrétion interne du testicule embryonnaire, en particulier de la glande interstitielle, oriente l'évolution masculine des gonocytes et du tractus génital somatique.

Le premier fait d'observation positif venu plaider en faveur de la théorie hormonale de la différenciation sexuelle a été apporté par l'étude des free-martins des Bovidés (Keller & Tandler, 1916; Lillie, 1916, 1917). Cette observation cruciale est bien connue, mais il n'est pas inutile d'en rappeler les résultats essentiels.

Le chorion de la Vache est extrêmement allongé dès les stades précoces du développement, et porte un placenta cotylédonné. Dans les grossesses gémellaires, les chorions viennent en contact et fusionnent la plupart du temps, au moins partiellement, sans doute au niveau de cotylédons placentaires. Il peut alors s'établir des connexions vasculaires précoces entre les fœtus, avant le stade de 15 mm. (Lillie, 1917). La dizygotie est établie par la présence de deux corps jaunes sur les ovaires maternels.

Or lorsque les deux embryons sont de sexe différent, le mâle est normal, mais la femelle (devenue le 'free-martin' des auteurs de langue anglaise) présente des signes évidents de masculinisation attribués à l'action d'une hormone produite par le mâle et parvenant à la femelle par voie sanguine. Cette interprétation est corroborée par le fait que, s'il ne s'établit pas d'anastomose vasculaire entre les chorions, la femelle reste normale.

Dans les free-martins, les ovaires sont stérilisés et le cortex ne se développe généralement pas; lorsqu'il apparaît, il présente des signes d'inhibition (Bissonnette, 1928*b*). La médulla peut s'hypertrophier et contenir de véritables tubes séminifères stériles. Les canaux de Müller ont plus ou moins régressé; les canaux de Wolff sont parfois développés sur toute leur longueur et portent des vésicules séminales peu volumineuses (cf. Bissonnette, 1924). Le vagin est souvent supprimé et la femelle porte un sinus urogénital indivis comme le mâle.

Mais les free-martins ne portent pas de prostate, même dans les cas extrêmes, et les organes génitaux externes gardent le type féminin. Le clitoris est parfois hypertrophié et constitue un organe pénisoïde mais non un vrai pénis (cf. Buyse, 1936).* Certains territoires échappent donc à la masculinisation qui frappe si nettement la plupart des structures internes. Lillie (1923) a pensé que l'organisme féminin exerce une certaine résistance à l'hormone mâle, peut-être en relation avec sa détermination génétique féminine.

Il faut remarquer que les cas de free-martins ne mettent aucune sécrétion ovarienne en évidence et ne démontrent pas d'une manière absolue l'intervention d'une hormone testiculaire. On pourrait songer à expliquer l'action d'un des deux sexes sur l'autre par des différences métaboliques générales, ou par des substances issues de l'ensemble du complexe somatique, plutôt que de sécrétions des gonades; Moore (1944) propose une telle interprétation dans un article mettant en doute l'exactitude de la théorie hormonale de la différenciation sexuelle.

D'autres observations faites sur les Mammifères soulèvent certaines difficultés à l'explication hormonale du free-martin. Si chez certains Mammifères, dont les chorions embryonnaires présentent parfois une fusion sans connexion vasculaire, les femelles restent normales, ce qui est en accord avec l'interprétation des free-martins (Chèvres, Moutons, étudiés par Lillie et Keller & Tandler), dans d'autres cas il existe entre les embryons des anastomoses circulatoires sans qu'aucun effet de free-martin ne s'ensuive: Wislocki & Hamlett (1934) ont signalé un cas chez le Chat et Wislocki (1939) a bien étudié l'Ouistiti à ce sujet.

D'autre part, Hughes (1929) a décrit des free-martins chez le Porc. Or l'hermaphrodisme vrai est particulièrement fréquent dans cette espèce; on comprend mal pourquoi, lorsque les gonades sont portées par deux individus différents, les testicules provoquent l'atrophie ou la masculinisation partielle des ovaires, alors qu'un même individu peut porter un ovaire et un testicule normaux.

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* Frazer & Greenwood (1928) ont signalé l'existence d'une prostate dans un free-martin âgé, mais sans examen histologique.

Il se peut que parfois la sécrétion des gonades foetales ne passe pas dans la circulation générale en quantité suffisante et exerce une action surtout localisée ou unilatérale. L'existence très générale des sécrétions génitales embryonnaires a en effet été établie expérimentalement comme nous on le constatera dans les pages suivantes.*

II. DÉMONSTRATION DE L'EXISTENCE DE SÉCRÉTIONS GÉNITALES EMBRYONNAIRES

On a démontré l'existence de sécrétions des gonades embryonnaires d'une manière particulièrement nette en utilisant les méthodes générales de l'endocrinologie qui consistent soit à faire agir la glande sur un organisme qui ne la porte pas (greffes, parabiose) soit à supprimer la glande par castration.

(1) *Greffes*

Les expériences les plus nombreuses ont été réalisées chez les Batraciens. La différenciation sexuelle de ces animaux est tardive: elle a lieu au cours des semaines qui suivent l'éclosion, au sein d'une larve qui mène une vie libre et n'est plus un embryon.

Batraciens. Après avoir réalisé sur l'Ambystome des greffes intra-cœlomiques de glandes génitales, Humphrey introduisit en 1929 la remarquable méthode des greffes orthotopiques: il excise dans une larve hôte très jeune (stade 24 à 32) la région du mésoderme contenant l'ébauche présomptive de la gonade et la remplace par un fragment identique prélevé sur un donneur. On peut élever les deux larves et en reconnaître le sexe. Le greffon se développe normalement et lorsque le donneur et l'hôte sont de sexe opposé ce dernier porte deux gonades de sexe différent. L'opération demande cependant à être faite avec grand soin, car si en préparant l'hôte on n'a pas enlevé totalement l'ébauche présomptive de la gonade, celle-ci peut se développer et risque de se mélanger avec le greffon.

Les résultats de ces expériences ont été très nets. Dans la majorité des cas le testicule modifie l'ovaire qui se trouve sur le même animal. L'ovaire est soit stérilisé soit transformé dans le sens testiculaire, au moins à certains niveaux: la médulla s'hypertrophie et le cortex est graduellement inhibé. L'inversion de l'ovaire en un testicule, qui commence en général après le début de la différenciation sexuelle, n'est pas totale; la gonade garde toujours certaines particularités qui font aisément reconnaître son origine ovarienne (Humphrey, 1929, 1931*a*). Cependant au moment de la maturité sexuelle on peut trouver des spermatozoïdes au moins dans une partie de l'ovaire masculinisé (Humphrey, 1931*a*), et ce sperme peut féconder les œufs d'une femelle normale (Humphrey, 1942*a*).

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* La théorie hormonale de la différenciation du sexe a fait l'objet d'un grand nombre d'articles; nous renvoyons le lecteur en particulier à ceux de Burns (1938*a*, 1942), de Willier (1939), de Witschi (1939), et de Wolff (1947).

Dans un petit nombre de cas c'est l'ovaire qui transforme le testicule: la transformation débute par la persistance d'un cortex ovarien et peut conduire à une glande présentant une structure ovarienne poussée. Il faut naturellement, au cours de telles recherches, tenir compte du type sexuel de la race de Batraciens étudiés: certaines races ont en effet des tendances hermaphrodites, et il existe normalement un cortex sur les testicules (Humphrey, 1933*b*; Witschi, 1933). Les cas de dominance femelle sont particulièrement nets dans certaines combinaisons hétéroplastiques. Ainsi Humphrey (1935) a pu greffer la région génitale d'*Ambystoma punctatum* sur une espèce plus petite et à croissance plus lente *A. Jeffersonianum*, ou sur une espèce plus grande et à croissance plus rapide *A. tigrinum*. Dans certaines de ces combinaisons, l'ovaire de l'espèce la plus grosse modifie aisément le testicule de l'espèce la plus petite.

En résumé, les magnifiques recherches de Humphrey, en accord avec les expériences de parabiose entre larves de Bactraciens, montrent bien que les gonades des deux sexes produisent des sécrétions morphogènes qui peuvent inverser le sexe de la glande génitale opposée.

Embryon de Poulet. Plusieurs auteurs tentèrent de vérifier la théorie hormonale de la différenciation du sexe en transplantant en position chorioallantoïdienne des ébauches de glandes génitales indifférenciées sur des embryons un peu plus âgés (Willier, 1927, 1933; Corinaldesi, 1927; Dennis, 1936).* Les résultats furent négatifs; indépendamment du sexe de l'hôte les greffons se développaient conformément à leur sexe génétique. Bradley (1941) utilise une technique différente; il introduit le greffon dans le cœlome d'embryons de Poulet ou de Canard bien plus jeunes (72 h. pour l'embryon de Poulet). Dans les combinaisons hétérosexuées le testicule est parfois modifié par l'ovaire, mais les résultats sont inconstants.

Récemment Wolff (1946) a réussi à réaliser un véritable free-martinisme chez l'embryon de Poulet, en portant dans le cœlome d'hôtes encore plus jeunes (50 h.), des gonades d'âge varié. Dans ces conditions les ovaires greffés féminisent légèrement les testicules des hôtes, ils provoquent la persistance d'une plage corticale plus ou moins étendue. L'action des greffons est assez localisée, manifestée surtout sur la partie du testicule la plus proche de l'ovaire (Fig. 1). Les testicules implantés de la même manière n'ont pas masculinisé les ovaires, mais ont inhibé les canaux de Müller des femelles. Ces expériences résolvent donc une question longtemps débattue, et montrent que les glandes génitales du Poulet produisent des sécrétions actives dans l'histogénèse génitale; la sécrétion dominante paraît être celle du sexe féminin hétéro-gamétique.

Mammifères. J'ai réalisé chez le Lapin des greffes testiculaires sur l'embryon femelle de 20 à 23 jours, soit sous la peau de la cuisse, soit dans la cavité générale (Jost, 1946*a*, 1947*c*, *f*). Au cours de ces premiers essais, le greffon a masculinisé l'hôte dans un seul cas, le plus jeune (hôte de 20 jours, date du début de la différenciation sexuelle somatique). Le testicule implanté sur le mésosalpinx droit a inhibé et supprimé l'oviducte de ce côté et a provoqué une persistance plus étendue des canaux de Wolff. La taille de l'ovaire droit est nettement réduite par rapport à l'ovaire gauche, qui est presque trois fois plus long. Les greffes faites sur des femelles de 23 jours n'ont pas eu d'effet: elles sont peut-être trop tardives; on constate en effet que des embryons mâles et femelles unis en parabiose à cet âge par fusion des cuisses, se différencient normalement (Jost, 1946 *a*) et d'autre part que la castration fœtale n'entraine plus que des effets restreints à ce stade. Dans les expériences actuelles j'opére sur des stades plus jeunes.

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* On trouvera les références dans le travail de Wolff (1946).

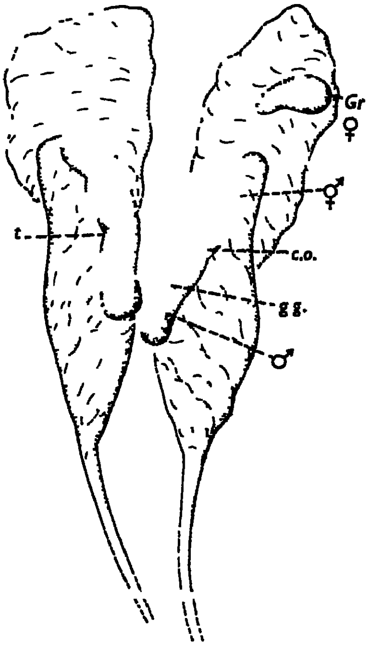

Fig. 1. Embryon de Poulet mâle portant une greffe ovarienne (*Gr.* ♀); la gonade droite de l'hôte est un testicule (*t*); la gonade gauche (*g.g.*) a la structure d'un ovotestis avec cortex ovarien (*c.o.*) dans la région antérieure, la plus proche du greffon, et d'un testicule dans la région postérieure. (Dessin aimablement communiqué par M. Et. Wolff, d'après Wolff, 1946, Fig. 10.)

(2) *Parabiose*

Introduite par Burns en 1925 dans l'étude des problèmes posés par le free-martin, la méthode de la parabiose entre larves indifférenciées de Bactraciens a été d'une remarquable fécondité entre les mains de Burns, Humphrey et Witschi (voir la synthèse de Witschi, 1939). Les larves sorties de la coque de l'œuf sont unies avant l'apparition de la motilité musculaire et peuvent être élevées ensuite plusieurs années.

(1) La sex ratio attendue de 50%♂♂+50%♀♀, les unions étant faites au hasard, a bien été observée par les différents auteurs à l'exception des premières données de Burns (1925) qui sont discutées (cf. Witschi, Gilbert & Andrews, 1931; Humphrey, 1932, 1936). Dans les paires uni-sexuées les deux conjoints se développent en général normalement. Il n'en est pas de même dans les paires hétéro-sexuées. Dans l'interprétation des résultats il est naturellement indispensable de tenir compte du type sexuel de la race étudiée (Witschi, 1933, 1939).

(2) Les deux parabiontes commencent généralement à se différencier conformément à leur sexe génétique; ultérieurement l'un des sexes peut modifier le développement de son partenaire. D'après Burns (1930, 1931, 1935) le sexe du parabionte dominé est fréquemment inversé dès le début de la différenciation, toute évolution conforme au sexe génétique est supprimée ('direct differentiation' opposée à 'delayed inversion'). Humphrey (1936) admet qu'il peut en être ainsi dans un petit nombre de cas, au moins dans certaines régions des gonades. Cette observation bien établie aurait un grand intérêt pour prouver que dès son origine la flexion sexuelle de la gonade est sous contrôle hormonal. Witschi & McCurdy (1929) ont fait une observation importante à ce point de vue, en notant des manifestations

d'antagonisme sexuel antérieures à la différenciation morphologique des gonades, dans des couples de *Triturus torosus*.

(3) Dans la grande majorité des cas le mâle est dominant, comme dans les free-martins naturels. Sous son influence les ovaires sont atrophiés et stérilisés ('free-martin effect' de Witschi) ou transformés dans le sens testiculaire (inversion du sexe) au moins dans une partie de son étendue (Witschi, 1927; Witschi & McCurdy, 1929; Burns, 1930; Humphrey, 1932, 1936).

(4) L'ovaire peut parfois être dominant; il en résulte dans le mâle une transformation des testicules en ovaires. La dominance femelle est extrêmement nette dans certaines combinaisons hétéroplastiques dans lesquelles on unit des espèces de taille et de vitesse de croissance différentes. Ainsi les unions *Ambystoma tigrinum* ♀ + *A. jeffersonianum* ♂, les testicules du mâle nain peuvent être transformés en ovaires (Witschi, 1937). Dans les unions *A. tigrinum* ♀ + *A. maculatum* ♂, la femelle de la grande espèce peut féminiser son conjoint si celui-ci n'est pas d'une race à tendance gonochorique trop tranchée. Le mâle peut au contraire dominer s'il est de race nettement différenciée; le membre dominant de la paire n'est donc pas celui qui se différencie le plus tôt, mais celui dont la sécrétion est la plus puissante (Witschi, 1937).

Les cas de dominance femelle montrent que l'organisme féminin produit également une substance morphogène au cours de sa différenciation sexuelle.

(5) Les interactions entre les parabiontes sont plus faibles et plus localisées chez les Anoures que chez les Urodèles (Witschi, 1931). Alors que chez ces derniers les inducteurs sexuels passent dans la circulation générale, leur action ne se fait sentir qu'à faible distance chez les Anoures, et Witschi admet qu'ils diffusent à travers les tissus. Pour que le testicule puisse modifier l'ovaire, il est nécessaire que les deux ébauches soient presqu'en contact, et seules les parties de l'ovaire les plus proches sont atteintes. Cette observation souligne d'une manière suggestive l'origine testiculaire de la substance inductrice. Chez le Crapaud l'action des inducteurs est si localisée qu'elle est impossible à mettre en évidence dans les expériences de parabiose.

(6) Witschi (1942) a insisté récemment sur la spécificité taxonomique des inducteurs sexuels. Dans les parabiontes de genres différents *Ambystoma* + *Triturus*, Witschi & McCurdy (1943) constatent que les gonades de *Triturus* sont très sensibles aux inducteurs sexuels des Ambystomes, mais ces derniers ne répondent jamais aux facteurs correspondants de *Triturus*; Witschi explique ce fait par une spécifité taxonomique des substances inductrices qui seraient des protéines.

(7) Dans certaines paires de parabiontes les ovaires de la femelle sont extraordinairement atrophiés (cas d'*Ambystoma tigrinum* ♀ + *A. jeffersonianum* ♀). Or au moment de la maturité sexuelle, dans ces femelles pratiquement castrées, le tractus génital somatique, est identique à celui des mâles normaux (Witschi, 1936*b*). Le tractus génital somatique des Batraciens est donc également sous le contrôle de sécrétions des glandes génitales.

(3) *Castration*

Les expériences de castration sont destinées à établir le rôle des gonades dans la différenciation du tractus génital somatique.

Batraciens. D'intéressantes expériences ont été effectuées par de Beaumont (1933) sur le Triton (*Triturus cristatus*). Dans de jeunes Tritons de 5 à 6 cm. les gonades sont déjà bien différenciées, mais les caractères sexuels somatiques sont encore indifférenciés. La castration est effectuée à ce moment.

Chez l'adulte les différences sexuelles sont moins marquées que chez les Mammifères. Les deux sortes de gonoductes persistent dans les deux sexes, mais un seul est bien développé. Les canaux collecteurs du rein s'ouvrent par un orifice distinct dans les papilles urogénitales du mâle, et débouchent au contraire dans la partie postérieure du canal de Wolff de la femelle. Enfin, alors que le mâle porte trois paires de glandes cloacales, la femelle n'en a qu'une.

Les Tritons castrés avant la flexion sexuelle somatique prennent un aspect 'neutre', identique quel que soit le sexe génétique. Dans la forme neutre les deux sortes de gonoductes restent frêles, les canaux collecteurs du rein et la papille cloacale ont un type intermédiaire entre le type mâle et femelle. Sur ces derniers caractères le testicule exerce une action stimulante et l'ovaire une action inhibitrice.

Lorsque l'on greffe des testicules ou des ovaires sur un castrat, la différenciation sexuelle est conforme au sexe des glandes greffées.

Enfin de Beaumont insiste sur un fait qui donne toute sa signification aux résultats précédents: la castration chez l'adulte ne peut plus conduire au type neutre, l'organisme garde des caractères masculins ou féminins, car le tractus génital a subi une détermination irréversible.

Mammifères. Moore (1941, 1943) a récemment étudié le rôle des gonades dans la différenciation sexuelle somatique en castrant de jeunes Opossums (*Didelphys virginiana*) contenus dans la poche marsupiale. Au moment de la naissance le petit Opossum est dans un état rudimentaire, et son sexe n'est pas encore indiqué. Les gonades subissent leur orientation sexuelle dans les premiers jours de la vie extra-utérine, et les premières différences somatiques ne se révèlent qu'à 9 ou 10 jours, à l'extrémité des canaux de Müller qui est abortive chez le mâle. Les bourgeons prostatiques apparaissent vers le 17^e jour, et dès ce moment les canaux de Müller du mâle sont bien plus grêles que ceux de la femelle; à 20 jours l'involution des canaux de Müller du mâle est en cours (cf. Burns, 1945).

Moore castre chirurgicalement les petits Opossums à l'âge de 20 jours ou plus. Il constate que malgré la gonadectomie la différenciation sexuelle s'opère normalement. Les canaux de Müller du mâle poursuivent leur involution et la prostate, en particulier, constituée à 20 jours par de simples excroissances de la paroi du sinus urogénital continue à se développer et à se ramifier. L'action des hormones sécrétées par les gonades ne se fait sentir que vers l'âge de 100 jours, et c'est à cette date que commencerait la sécrétion des glandes génitales.

Moore estime que ces résultats démontrent que la différenciation sexuelle somatique ne dépend ou ne nécessite pas de sécrétions des gonades.*

Cette conclusion déniant tout rôle aux sécrétions génitales dans la différenciation du sexe de l'Opossum est exactement opposée à celle à laquelle m'a conduit l'étude de la castration des fœtus de Lapin (Jost, 1946*c*, 1947 *a*, *e*, *f*). Ici la gonade acquiert ses caractéristiques sexuelles entre 14 et 15 jours, et la différenciation sexuelle somatique commence à l'âge de 20 jours (cf. Jost, 1947). A cet âge les canaux de Müller du mâle bien qu'encore continus commencent leur régression, qui aura lieu les jours suivants. Les premiers bourgeons prostatiques se développent vers 21 jours. La femelle garde ses canaux de Wolff jusqu'à l'âge de 24 jours; ils disparaissent ensuite, et la différenciation sexuelle est pratiquement terminée deux à trois jours avant la naissance qui a lieu à 30 jours ou un peu plus.

On castre l'embryon intra-utérin en extrayant la partie postérieure de l'embryon hors de l'utérus. Chaque gonade est enlevée par une incision dorso-latérale, puis les plaies sont recousues et le fœtus est remis en place dans l'utérus (technique décrite dans Jost, 1947*f*). Naturellement la glande prélevée est étudiée histologiquement; elle indique le sexe du fœtus. Les castrations ont été effectuées entre 19 et 24 jours et les effets de l'opération dépendent étroitement de sa date.

Dans les embryons castrés avant le début de la différenciation sexuelle somatique (à 19 jours) et étudiés peu avant le terme, aucun caractère masculin ne se différencie. Les canaux de Wolff ont totalement disparu, comme dans une femelle; aucun bourgeon prostatique ne s'est développé (Fig. 3A), et les organes génitaux externes ont un aspect féminin, la lame balano-préputiale n'entourant pas complètement l'urètre comme c'est le cas dans les mâles (Fig. 4).

De plus, les mâles castrés gardent leurs canaux de Müller qui se différencient en vagin müllérien (organe très développé chez la Lapine), cornes utérines et parfois oviductes; mais ces organes ont un diamètre plus faible que dans une femelle normale (Fig. 2).

Ainsi la castration précoce de l'embryon mâle empêche l'apparition de tout caractère sexuel masculin, et détermine une féminisation du tractus génital. J'ai expliqué ces faits (Jost, 1947*a*) en admettant que le développement de la prostate et des organes génitaux externes du mâle sont conditionnés par la sécrétion testiculaire et que les deux sortes de gonoductes n'ont pas les mêmes potentialités: le canal de Wolff régressant, comme le corps de Wolff, s'il n'est pas maintenu et stabilisé par la sécrétion du testicule embryonnaire, et le canal de Müller persistant au contraire, quel que soit le sexe génétique de l'embryon, s'il n'est pas inhibé par ce testicule.† Cette hypothèse est d'ailleurs corroborée par le résultat de la castration de l'embryon femelle.

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* 'The differentiation of these reproductive tracts (the male or female reproductive system) does not depend upon, or require, the presence of gonadal secreted hormones' (Moore, 1943, p. 453).

† La différence notée entre les deux sortes de gonoductes s'explique-t-elle par l'intervention de sécrétions maternelles ou d'autres glandes fœtales? Cette hypothèse devra être vérifiée, mais semble peu probable; rappelons en particulier que dans des greffes du tractus génital d'embryons sur des Rats impubères, Moore & Price (1942) ont également noté que le canal de Müller se développe

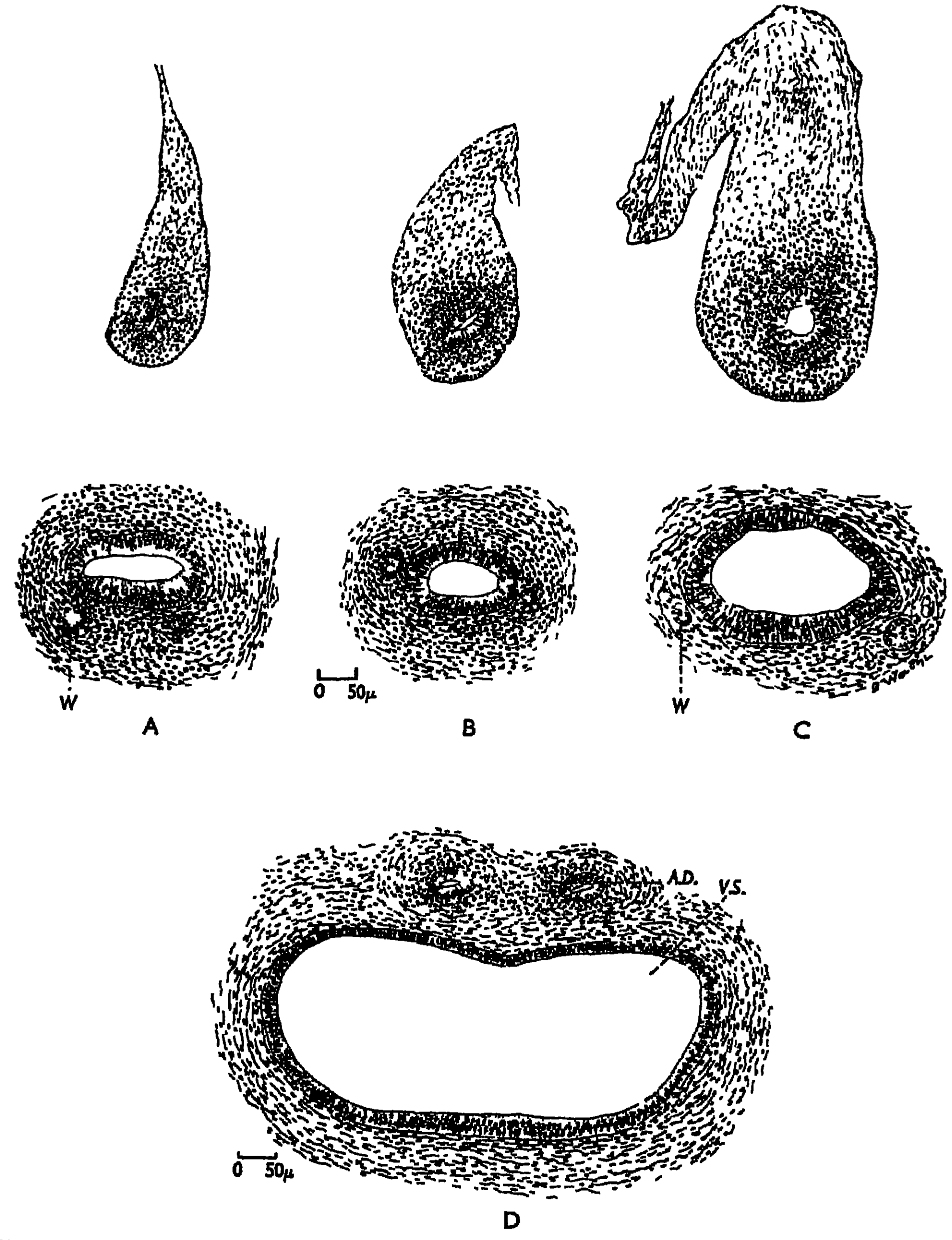

Fig. 2. Coupes à travers le tractus génital de quatre fœtus de Lapin de la même portée, sacrifiés à 26 j. 20 h. Corne utérine et vagin müllérien d'un mâle castré à 20 j. 20 h., d'une femelle castrée au même stade et (C) d'une femelle normale. Il persiste encore de très faibles restes wolffiens (*W*) dans la partie inférieure du cordon génital à l'âge où ont été sacrifiés les fœtus. En D, la vésicule séminale (*V.S.*) et les ampoules déférentielles (*A.D.*) d'un mâle normal; ces organes wolffiens occupent la même situation que le vagin müllérien des femelles ou du mâle castré.

généralement, alors que le canal de Wolff a une forte tendance à disparaître. Mais ces auteurs enregistrent le même résultat lorsque le testicule était transplanté avec les gonoductes: il se peut que dans les conditions de la greffe, il ait été mis dans l'impossibilité d'agir sur les conduits sexuels.

La castration du mâle effectuée peu après le début de la différenciation sexuelle (20 ou 21 jours) a des conséquences comparables à celle d'une gonadectomie plus précoce, mais n'empêche pas l'apparition de deux bourgeons prostatiques peu développés et qui correspondent au lobe antérieur de la prostate, le premier à se différencier normalement (Fig. 3B).

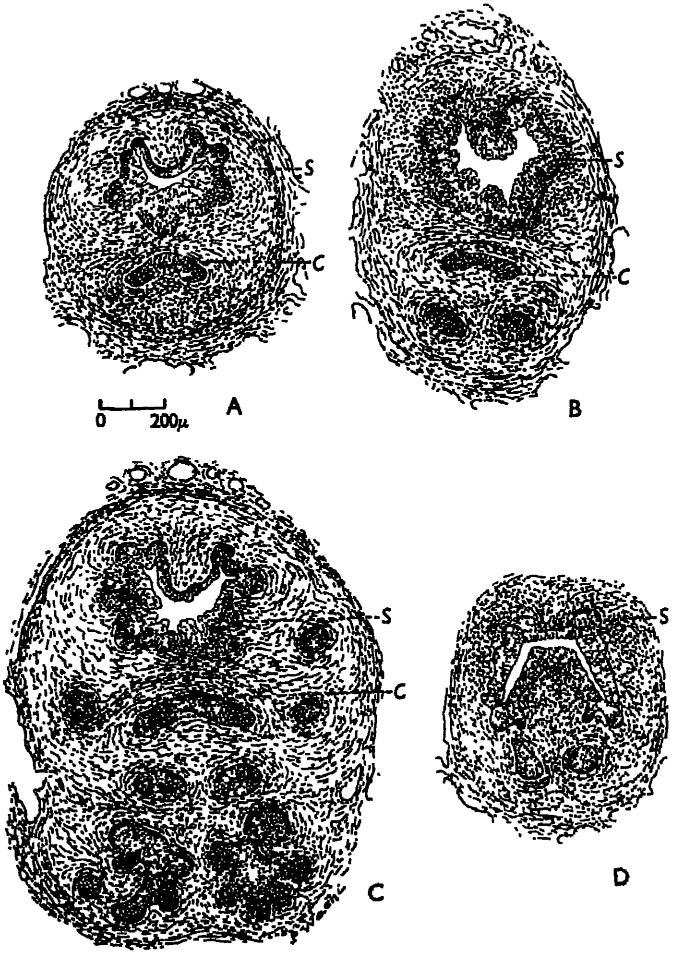

Fig. 3. Coupes à travers la région prostatique de divers embryons mâles de Lapin: A: mâle de 28 j., castré à 19 j.; aucun bourgeon prostatique. B: mâle de 28 j., castré à 21 j.; 2 bourgeons prostatiques. C: mâle de 28 j., castré à 23 j.; la prostate particulièrement développée dans ce cas est comparable à celle d'un mâle normal. D: mâle témoin de 23 j.; niveau situé un peu en arrière des précédents pour mieux montrer les bourgeons prostatiques. [Ne pas confondre avec un bourgeon prostatique le cordon sinusaire (*C*) qui relie la partie inférieure des gonoductes au sinus urogénital (*S*).]

Lorsque la castration a lieu à 23 jours le résultat en est très différent. Les canaux de Müller dont la régression était déjà très avancée continuent à disparaître et la partie postérieure des canaux de Wolff, correspondant à la vésicule séminale, persiste, mais le canal déférent régresse. D'autre part, la prostate, formée seulement de quelques bourgeons simples à 23 jours, continue à proliférer et à se ramifier, cependant un peu moins, en général, que dans les mâles témoins (Fig. 3).

Dans les embryons castrés encore plus tard, à 24 jours, les différents caractères sexuels masculins continuent à se différencier dans le sens mâle, mais la prostate semble réduite par rapport aux témoins (Jost, 1947*e*, *f*).

Les effets de la gonadectomie doivent bien être attribués à une déficience humorale et non au traumatisme ou à des causes non spécifiques. Dans les mâles castrés unilatéralement entre 19 et 23 jours la prostate et les organes génitaux externes se différencient normalement et le canal de Wolff peut être maintenu du côté castré par le testicule situé du côté opposé; il aurait régressé si la castration avait été totale, et n'a pu être préservé par le testicule unique qu'au moyen d'une sécrétion parvenue jusqu'à lui. Dans d'autres cas, après castration unilatérale précoce, l'action du testicule unique est plus localisée ou unilatérale et le canal de Wolff ne persiste pas intégralement du côté castré; l'utérus peut même subsister de ce côté n'étant pas suffisamment inhibé, mais la prostate et les organes génitaux externes sont bien développés (Jost, 1947*e*, *f*).

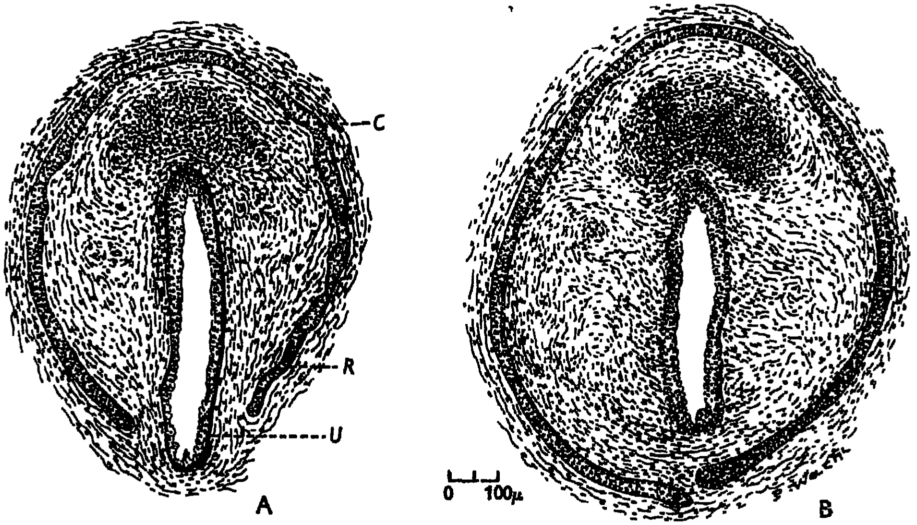

Fig. 4. Coupes à travers les organes génitaux externes de fœtus de Lapin, au stade de 28 j.: A, mâle castré à 21 j.; B, mâle castré au même âge mais ayant reçu une implantation de 0·5 mg. de méthyl-testostérone; ce dernier a l'aspect masculin typique. *C*, corps caverneux; *R*, repli balano-préputial; *U*, urètre.

D'autre part en implantant au moment de la castration un cristal d'androgène synthétique à la place des testicules, on assure le développement des caractéristiques sexuelles masculines dans les castrats. Les canaux de Wolff persistent et se différencient, la prostate et les organes génitaux externes sont parfaitement développés (Fig. 4). Cependant dans la première série d'expériences réalisées jusqu'à présent les canaux de Müller n'ont pas été inhibés d'une manière totale; ce point mérite d'être étudié en détail, car il permettra de comparer les propriétés de la sécrétion du testicule embryonnaire et des androgènes de synthèse.

En ce qui concerne l'ovariectomie de la femelle, nous avons opéré à deux stades, à 23 jours et 21 jours. Les femelles castrées à 23 jours continuent à se différencier d'une manière normale; les canaux de Wolff, en particulier, régressent complètement. Les femelles ovariectomisées à 21 jours ont acquis une structure féminine également, mais le diamètre des organes müllériens est réduit par rapport à celui des mêmes organes dans une femelle normale (Fig. 2). Il semble donc que l'ovaire produit également une sécrétion durant la différenciation sexuelle, mais le testicule fœtal joue un rôle essentiel dans l'apparition des différences qui distinguent les deux sexes. Le développement des voies müllériennes des femelles castrées ne doit probablement pas être attribué à l'effet d'une stimulation ovarienne antérieure à l'ovariectomie, puisque les canaux de Müller persistent également dans les mâles castrés.

Le testicule n'a pas besoin d'agir sur les organes durant toute leur organogenèse pour en assurer la différenciation. Ainsi la vésicule séminale peut s'édifier dans un embryon castré à 24 jours dans lequel elle n'a fait qu'amorcer sa différenciation; la prostate peut après le 23^{e} jour continuer à pousser malgré la gonadectomie (Fig. 3). Une castration trop tardive ne peut plus empêcher le développement des structures qui sont déjà déterminées.

A la lumière de ces résultats on peut se demander si les gonadectomies effectuées par Moore sur l'Opossum n'ont pas été trop tardives. A 20 ou 22 jours la différenciation sexuelle des petits Opossums est bien avancée: la prostate est apparue depuis plusieurs jours et les canaux de Müller sont en involution. Les bourgeons prostatiques simples et courts à 20 ou 22 jours ont continué à se ramifier dans les castrats. On note un résultat analogue chez l'embryon de Lapin castré à 23 jours, mais dans cette espèce une castration plus précoce, antérieure à la différenciation sexuelle, supprime complètement tout développement prostatique. Il semble donc nécessaire de castrer les Opossums plus jeunes avant d'admettre que les gonades ne jouent aucun rôle dans l'histogenèse génitale.

Moore ne note pas de réduction sensible et constante du développement prostatique des castrats par rapport aux témoins, mais il n'a pas fait d'étude quantitative systématique. Dans un cas dont l'étude histologique ne révélait pas de déficience, les mesures planimétriques mettent en évidence une réduction de la masse prostatique à 71% par rapport à un frère de la même portée. Une étude quantitative complète est donc nécessaire pour préciser l'effet de la castration sur les annexes. Une telle recherche a été effectuée par Wells (1946*b*) qui réalisa des castrations sur l'embryon de Rat selon la technique, et se developpe dans l'abdomen: l'embryon à opérer est sorti de l'utérus auquel il reste relié par le placenta suivante. Cette méthode de permet malheureusement que des interventions tardives, 50 à 60 heures avant le part, et sans effet sur l'organogenèse propement dite. Malgré la briéveté de ce délai, Wells observe, grâce à des mesures quantitatives, une nette réduction de volume des glandes annexes.

(4) *Extraction chimique des hormones*

Si les gonades embryonnaires produisent des hormones on doit pouvoir les extraire grâce aux méthodes biochimiques. Pour être tout à fait démonstrative une telle étude doit déceler la substance d'une manière assez élective dans l'organe qui la produit et prouver son activité spécifique sur un test embryonnaire défini. Les recherches effectuées jusqu'à présent n'ont pas encore pu répondre à des exigences aussi strictes.

Womack & Koch (1932) montrent que les testicules de fœtus de Veau renferment des androgènes actifs sur la crête de Coq; mais ils n'indiquent pas l'âge des fœtus. Atsumi (1939) extrait du testicule et de l'artère ombilicale de fœtus de Veau des androgènes actifs sur des souris mâles. Mais ces androgènes n'apparaissent qu'à partir du stade de 60 cm. (6^e^ mois); or la différenciation sexuelle des embryons a lieu à des stades antérieurs à 15 cm. environ (Bissonnette, 1924). Ces expériences ne permettent donc pas de conclusions nettes en ce qui concerne l'activité testiculaire dans la différenciation sexuelle; l'étude des stades jeunes est extrèmement difficile car elle nécessite un nombre impressionnant de glandes minuscules.

De son côté Raynaud (1942) prépare un extrait à partir de testicules d'embryons de Veau de 20 à 60 cm., et l'injecte à des souris gravides. Il observe un pouvoir légèrement masculinisant de ces extraits sur l'épididyme des embryons femelles. Dans trois femelles l'ovaire présentait certaines anomalies qui témoignent peut-être de l'activité des extraits. Ces intéressantes recherches sur l'embryon méritent d'être reprises et étendues.

(5) *Action des hormones sexuelles sur l'embryon*

Le fait que l'on puisse inverser totalement le sens de la différenciation du sexe sous l'action d'une hormone sexuelle apporte sinon la preuve de l'intervention normale d'hormones dans l'organogenèse génitale, au moins un argument important. Mais les cas dans lesquels les hormones sont capables d'induire une histogenèse opposée au sexe génétique mais d'apparence normale, sont encore rares. Nous ne citerons que quelques exemples ici (revue dans Jost, 1947*f*).

Chez les Batraciens, le propionate de testostérone (voir en particulier Gallien, 1937, 1941, 1944) ou la prégnèninolone (Eversole & d'Angelo, 1942; Jost, 1943) administrés avant la différenciation sexuelle peuvent masculiniser totalement les gonades de femelles génétiques; or l'histogenèse masculine de ces têtards reproduit d'une manière identique les processus normaux de la différenciation des mâles (Witschi, 1942). Inversement, dans certaines conditions les œstrogènes féminisent les têtards mâles (voir en particulier Burns, 1938*b*; Gallien, 1941).

Chez l'embryon de Poulet, la folliculine introduite dans l'œuf durant les premiers jours de l'incubation féminise les mâles génétiques (Willier, Gallagher & Koch, 1935; Dantchakoff, 1935; Wolff & Ginglinger, 1935). Pour une dose suffisante les mâles peuvent acquérir une structure féminine typique. Lorsque la dose est insuffisante ou le traitement trop tardif (Et. & Em. Wolff, 1947) les mâles sont seulement légèrement féminisés et portent un cortex rudimentaire à la surface de leurs testicules. Or Wolff obtient la même transformation en greffant précocement un ovaire embryonnaire sur d'autres mâles. De son côté, le propionate de testostérone ne masculinise que faiblement les ovaires des femelles, mais provoque la régression (ou inhibition de developpment des canaux de Müller des femelles (Willier, Rawles & Koch, 1938; Dantchakoff, 1938). Or des greffes de testicules embryonnaires agissent de même sur les embryons femelles (Wolff, 1947).

Chez les Mammifères, aussi bien les Marsupiaux (Burns, 1939, 1942; Moore, 1939) que les Placentaires (Dantchakoff, 1937*b*, 1947; Greene, Burrill & Ivy, 1939*a*, 1940; Raynaud, 1938, 1942; Turner, 1939; Jost, 1947*f*), les hormones sexuelles modifient le tractus génital somatique mais n'inversent pas les gonades. Elles n'induisent pas non plus la réalisation d'un appareil génital absolument normal: les androgènes masculinisent les femelles en provoquant le développement des organes qui chez l'adulte sont sous le contrôle testiculaire (canaux de Wolff, prostate, organes génitaux externes), mais n'inhibent pas les voies müllériennes. Les œstrogènes provoquent outre une certaine féminisation des mâles, des réactions plus complexes parfois 'paradoxales'. Mais si l'on s'en tient à l'étude de certaines structures comme les organes génitaux externes, les hormones sexuelles sont capables d'en diriger la différenciation, exactement comme le déterminisme génétique (*v.* l'étude récente de Bruner & Witschi, 1946, sur le Hamster).

III. RÔLE DES AUTRES GLANDES ENDOCRINES DANS LA DIFFÉRENCIATION SEXUELLE

Nous n'avons envisagé jusqu'à présent que les glandes génitales. D'autres glandes jouent-elles un rôle dans la différenciation sexuelle? On songe naturellement surtout à l'hypophyse, à la surrénale et à la thyroïde.

Hypophyse. Plusieurs séries d'expériences réalisées sur les Batraciens ont montré que l'hypophysectomie précoce des têtards n'empêche pas la différenciation sexuelle des gonades, ni le développement ultérieur de celles-ci, au moins jusqu'à un stade déterminé (Allen, 1917; Atwell, 1933; Humphrey, 1942*b*). Humphrey (1933*c*) prouve que la sécrétion des gonades n'est pas tarie dans les *Amblystoma tigrinum* hypophysectomisés précocement, en constatant l'inhibition ovarienne par le testicule situé dans le même individu après greffe orthotopique.

L'hypophysectomie de l'embryon de Poulet, effectuée par irradiation aux rayons X (Ancel, 1937; Wolff & Stoll, 1937) ou par la voie chirurgicale (Fugo, 1940), n'altère pas non plus la différenciation sexuelle. Fugo constate cependant, contrairement aux autres auteurs, que dans la deuxième moitié de l'incubation les embryons hypophysectomisés ont une taille réduite et que leurs gonades sont un peu plus petites que normalement. Chez l'embryon de Souris irradié Raynaud & Frilley (1947) notent que l'hypophyse n'est pas indispensable à l'organogenèse génitale. J'étudie actuellement ce problème sur le Lapin à l'aide d'une technique qui permet une hypophysectomie absolument totale et à des dates variables: le fœtus est décapité et poursuit son développement sans tête (Jost, 1947*d*). Les résultats histologiques seront publiés ultérieurement. Dans certaines de mes expériences de castration incomplète et précoce sur le Lapin, la diminution du volume testiculaire paraît avoir été suivie d'une diminution de la quantité de sécrétion; il ne semble pas y avoir eu d' 'hypertrophie sécrétoire compensatrice'. Il se peut que cette observation soit à rapprocher des précédentes.

Il semble donc que l'hypophyse ne joue pas de rôle sensible dans la différenciation sexuelle et que les gonades sont capables de produire leur sécrétion en absence de stimulation hypophysaire.* Cependant certains auteurs, opérant généralement en fin de gestation, ont noté l'hypertrophie testiculaire sous l'action d'une gonadotrophine exogène (e.g. Aron, 1933; Wells, 1946*a*). Aron signale même que dans des embryons de Cobaye de 80 mm. le traitement a entraîné une hypertrophie des vésicules séminales. Il serait intéressant de préciser les réactions aux gonadotrophines du testicule d'embryons plus jeunes, au moment où a lieu la différenciation sexuelle.

D'autres auteurs ont dosé le contenu gonadotrope de l'hypophyse fœtale: chez le Porc, Smith & Dortzbach (1929) décèlent des gonadostimulines, vers la fin de gestation (stades de 18 à 20 cm. et plus). L'hypophyse fœtale de Cheval possède d'après Hellbaum (1935) un pouvoir gonadotrope plus faible à poids égal, que celle de l'adulte, mais sensible dès le stade de 20 cm. Naturellement trouver des gonadostimulines dans l'hypophyse ne suffit pas à démontrer qu'elles sont normalement sécrétées et jouent un rôle physiologique.

Surrénales. Divers auteurs ont mis en évidence l'activité androgène de la corticosurrénale dans certaines espèces, par exemple chez le jeune Rat (consulter à ce sujet Burrill & Greene, 1940). La surrénale embryonnaire n'aurait-elle pas une activité androgène comparable? On a posé le problème, en particulier à propos des résultats négatifs des expériences de castration effectuées par Moore; on pourrait supposer que le testicule a été suppléé par la surrénale fœtale. Chez l'embryon mâle de Lapin castré suffisamment tôt, les surrénales ne jouent pas de rôle vicariant décelable (Jost, 1947*f*). Opérant à la fin de la vie embryonnaire Wells (1946*b*) compare les annexes d'embryons de Rats normaux, castrés, ou castrés et surrénalectomisés, et conclut: 'if the adrenal of foetal male produce any such hormone, the quantity is not sufficient to prevent the effects of castration upon the accessory reproductive organs.' D'autres recherches sont nécessaires pour préciser le rôle possible des surrénales dans la différenciation du sexe, mais il semble que ce rôle ne puisse être que secondaire. Ajoutons que Carnes (1940) n'a pu déceler d'activité androgène dans les surrénales de fœtus humains de 18 à 40 semaines (si elle existe, l'activité est inférieure à 10 U.I. par kg. d'organe frais). Enfin, il y aurait lieu d'étudier l'activité œstrogène possible de la surrénale fœtale.

Thyroïde. La thyroïdectomie précoce n'empêche pas la différenciation sexuelle normale des glandes génitales de têtards d'*Ambystoma* ou de *Rana* (Allen, 1918; E. R. & M. M. Hoskins, 1919). Les Hoskins notent que dans les stades ultérieurs du développement les oviductes ne se développent pas. L'agénésie thyroïdienne accompagnant celle de l'hypophyse est absolument sans effet sur le développement génital de l'embryon de Poulet d'après Ancel (1937) et Stoll (1939).

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* Voir l'Addendum à ce sujet.

IV. MODE D'ACTION ET NATURE DES SÉCRÉTIONS GÉNITALES EMBRYONNAIRES

Le sexe génétique se manifeste tardivement au cours de l'embryogenèse, et le développement génital de tout individu comporte trois phases prédominantes successives qui sont: (1) différenciation sexuelle des gonades, (2) différenciation sexuelle somatique, (3) épanouissement des caractères sexuels secondaires à la puberté. On a pu démontrer à la suite d'extractions chimiques des gonades elles-mêmes, et de suppléance de ces gonades après castration, l'intervention des hormones sexuelles dans la troisième de ces phases. Nous ne connaissons sans doute pas encore les associations moléculaires de ces stérols sous leur forme physiologiquement active, ni le nombre exact des stérols qui interviennent effectivement dans la réponse normale des récepteurs, mais il est hors de doute que les hormones sexuelles chimiquement bien connues jouent un rôle essentiel dans la physiologie génitale de l'adulte. Nous ne sommes pas encore aussi bien renseignés en ce qui concerne les sécrétions embryonnaires. Pour Witschi (cf. 1931, 1934, 1936*a*, 1942) il existe une différence fondamentale entre les substances inductrices de la différenciation sexuelle de la gonade, probablement protéiques et les hormones sexuelles qui dirigent la différenciation sexuelle somatique et l'épanouissement des caractères sexuels secondaires. Outre ces deux groupes de substances, Witschi fait encore intervenir d'autres inducteurs tels que les inducteurs de la formation de l'oviducte.

D'autres auteurs admettent l'identité entre les sécrétions de l'embryon et de l'adulte (Dantchakoff, 1937*a*, 1947). Pour Wolff, le rôle des hormones sexuelles que l'on avait réduit au contrôle des caractères sexuels secondaires est prédominant même dans la différenciation primaire des sexes, et cet auteur exprime récemment sa pensée en écrivant: 'il n'y a pas de différence de nature entre les hormones sexuelles de l'adulte et les organisateurs de la différenciation sexuelle de l'embryon. Les unes et les autres appartiennent au groupe des stérols...' mais 'est-ce à dire que, dans une espèce déterminée l'hormone primaire de l'embryon est identique à l'hormone sexuelle de l'adulte? Nous ne saurions encore l'affirmer d'une manière absolue' (Wolff, 1947). Ainsi exprimée, cette opinion ne s'oppose pas d'une manière irréductible à celle des auteurs pour qui les facteurs embryonnaires se distinguent de ceux de l'adulte, à condition de les classer tous parmi les stérols.

Il est utile de rappeler, avant d'entrer dans les détails, que les glandes génitales embryonnaires, aussi bien en ce qui concerne leur propre différenciation, que dans leurs actions sur les voies génitales, semblent pouvoir produire leur sécrétion en l'absence de stimulation hypophysaire (cf. p. 214) contrairement aux gonades adultes. Il ne serait pas illogique de supposer qu'à ce mode de fonctionnement particulier pût correspondre une sécrétion particulière.

D'autre part, dans toute la discussion du problème envisagé ici, il est essentiel de retenir que reconnaître à une substance le pouvoir d'induire une histogenèse d'apparence normale, ce n'est pas prouver que cette substance soit l'agent physiologique actif dans l'embryon. Mais au contraire, il y a de fortes chances pour que cette substance ne soit pas l'agent physiologique de l'embryon, si placée dans les meilleures conditions requises, elle reste incapable de suppléer la sécrétion de la glande fœtale.

(1) *Différenciation sexuelle des gonades*

(*a*) *Mode d'action des inducteurs sexuels de la gonade.* En 1903 Bouin et Ancel remarquaient que les gonades ont déjà acquis leur structure caractéristique avant que les cellules germinales ne présentent aucune marque sexuelle distinctive. La différenciation des parties somatiques de la gonade précède celle des gonocytes et orienterait l'évolution de ces derniers. Cette observation est en accord avec la théorie d'Ancel (1902, 1935) sur le rôle des tissus environnants dans l'orientation sexuelle des gonocytes.*

De son côté Witschi (1914) note que la différenciation sexuelle des gonocytes de la Grenouille résulte d'une induction: quel que soit leur sexe génétique, les cellules germinales placées dans le cortex évoluent en ovogonies, celles qui se trouvent dans la médulla donnent des spermatogonies.

Humphrey (1933*a*) a réalisé une belle expérience sur des larves de *Rana sylvatica* en extirpant l'ébauche présomptivede la gonade avant que les gonocytes ne soient venus la peupler et en la remplaçant par un fragment identique prélevé sur une autre larve. Les deux gonades se différencient suivant leur sexe génétique et sont donc de sexe différent dans 50% des cas. Or ce sont les cellules de l'hôte qui viennent habiter les deux gonades, et qui se différencient en spermatogonies dans le testicule, en ovogonies dans l'ovaire.

La bipotientialité des cellules germinales est également illustrée par les expériences de changement de sexe chez l'embryon, ou même après la naissance: chez la Poule après ablation de l'ovaire fonctionnel gauche, la gonade droite évolue en un testicule qui peut contenir des spermatozoïdes; chez le Crapaud mâle castré l'organe de Bidder prend un développement ovarien.

La différenciation sexuelle des gonades commence donc par celle de leurs constituants somatiques. C'est au moment où se précise le sexe des glandes génitales que se manifeste l'action des facteurs génétiques du sexe. La transformation de l'ébauche indifférenciée en une glande sexuée est-elle imposée directement par la constitution chromosomique de chaque sexe, l'assortiment génique jouant un rôle morphogène local, ou bien est-ce sous la conduite directrice d'une commande humorale que débute cette transformation? Les free-martins ne permettent pas de répondre à cette question puisque le plus jeune connu a 3·2 cm. (Bissonnette, 1928*a*) alors que la différenciation de la gonade débute au stade de 2·5 cm. C'est à Witschi & McCurdy (1929) que l'on doit l'observation la plus importante dans ce débat, puisque ces auteurs ont noté des manifestations d'antagonisme sexuel antérieures à toute différenciation morphologique dans des couples parabiotiques de *Triturus torosus*.

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* Dantchakoff (1947) n'admet pas cette manière de voir; pour elle les gonocytes 'sont dès l'abord orientés vers une destinée mâle chez les uns, femelle chez les autres'.

La conception défendue depuis de longues années par Witschi permet de comprendre le mécanisme de la flexion sexuelle de la gonade. Les deux constituants de la gonade indifférenciée, cortex et médulla, seraient des inducteurs antagonistes libérant chacun une sécrétion ayant un double effet: la 'cortexine' oriente les gonocytes dans le sens féminin, et inhibe la médulla; la 'médullarine' a l'action inverse (Witschi, 1931). C'est la prédominance de l'un de ces systèmes inducteurs qui oriente la flexion sexuelle de la gonade dans un sens ou dans l'autre. Ultérieurement Witschi (1934, 1942) a pensé que l'action stimulante positive sur les gonocytes et inhibitrice ou négative sur l'inducteur antagoniste pourrait être le fait de substances différentes, la corticine + et la corticine −, ou la médullarine + et la médullarine −. Comment s'établit la prédominance de l'un des inducteurs sur l'autre? Witschi pense surtout à des différences dans la quantité de sécrétion. Récemment P. Ancel (1946) a émis une hypothèse différente; le seul effet des gènes sexuels serait de rendre les tissus de l'un des constituants de la gonade plus sensibles à la sécrétion sexuelle opposée. La dominance de l'un des sexes ne serait pas due à des différences de la quantité de sécrétion produite par les deux sexes, mais à une inégalité de sensibilité. Padoa (1947) a mis en évidence une telle différence de sensibilité à l'action masculinisante du propionate de testostérone des gonades des deux génotypes mâle ou femelle d'une race indifférenciée de *Rana dalmatina*.

Il est important de retenir que le type sexuel acquis sous l'action des inducteurs corticaux et médullaire n'est pas irréversible. Chez certains Poissons ou Batraciens le sexe de la gonade déjà différenciée peut être inversé sous l'action des hormones sexuelles (voir en particulier Witschi & Crown, 1937; Foote & Witschi, 1939; Gallien, 1944). D'autre part un ovaire d'Ambystome en grande partie masculinisé par une greffe testiculaire orthotopique peut reprendre une structure ovarienne (Humphrey, 1931*b*). Dans le cas de parabiose *Ambystoma tigrinum* ♀ × *A. Jeffersonianum* ♂ les gonades féminisées du mâle nain peuvent faire retour à leur sexe génétique après la puberté (Witschi, 1937); ce cas est comparable à ceux déjà rapportés par Witschi (1936*a*) sous le titre: 'On the separate chemical control of embryonic sex differentiation and of secondary sex characters', et donne à l'auteur une raison puissante de penser que les substances inductrices de la différenciation sexuelle des gonades sont différentes des hormones sexuelles, puisque celles-ci sont incapables de maintenir les modifications induites par celles-là.

On peut rapprocher de cette observation celles de Wolff (1936) et de Dantchakoff (1936*a*) qui constatent que les gonades des Poulets féminisés par l'œstradiol font retour à leur sexe génétique même si le traitement à l'œstradiol est continué; celles de Gallien (1944) qui note que la folliculine est incapable de s'opposer à l'évolution masculine des ovaires que portent au moment de la métamorphose des mâles d'une race indifférenciée de Grenouilles ou des mâles génétiques féminisés au préalable par l'œstrogène. La structure de la gonade reste potentiellement bisexuée dans les cas précédents, l'ovaire renferme assez de matériel médullaire pour pouvoir se transformer en testicule. Les testicules induits chez les Batraciens par la testostérone sont stables (Gallien, 1944), probablement parcequ'il ne leur reste plus trace de cortex.

Les modifications des conduits génitaux sont au contraire définitivement acquises et irréversibles (Wolff, 1936, 1938; Greene & Ivy, 1937; Raynaud, 1938, 1942); ainsi dans un Coq dont l'ovaire induit par la folliculine se transforme en testicule, l'oviducte persiste toute la vie durant (Wolff, 1938). Un gonoducte qui a été stabilisé dans l'embryon ne régresse plus; celui qui a été inhibé ne peut plus se reconstituer. Signalons à ce sujet que le type sexuel des organes génitaux externes des Mammifères adultes dont on a beaucoup étudié la 'masculinisation' il y a une vingtaine d'années, gardent en réalité toujours une structure fondamentale irréversible, acquise dès la vie embryonnaire. On peut inverser le sexe de ces organes chez l'embryon, mais, chez l'adulte, si l'on parvient encore à modifier le clitoris en un organe pénisoïde, on ne le transforme plus en un pénis (cf. Raynaud, 1942; Jost, 1944).

(*b*) *Nature des substances inductrices de la gonade.* Comme on vient de le voir pour Witschi il s'agit de substances particulières, sans doute de nature protéique, différentes des hormones sexuelles et sans action sur la différenciation des voies génitales. Ces différences entre substances inductrices et hormones sexuelles expliqueraient le retour au sexe génétique que l'on observe généralement vers le moment de la puberté, dans les individus dont le sexe a été inversé, au cours des expériences de greffes ou de parabiose (Witschi, 1936*a*, 1937). Dans des *Ambystoma tigrinum* femelles ayant reçu une greffe testiculaire peu après la différenciation sexuelle Humphrey (1941) n'observe pas une telle disparition de l'inhibition ovarienne, même dans la deuxième année qui suit la maturité sexuelle. Mais les ovaires ne sont ni inversés ni stérilisés comme lorsque la greffe peut agir dès les premiers stades du développement.

Le principal argument parlant en faveur de la nature protéique des inducteurs réside dans les résultats de la parabiose entre genres différents d'Urodèles (Witschi & McCurdy, 1943). L'indifférence des Ambystomes aux inducteurs de *Triturus* est expliquée par une spécificité taxonomique des substances inductrices qui rend leur constitution protéique probable. Comme le fait remarquer Wolff (1947), une telle spécificité pourrait se comprendre également si l'inducteur était constitué par l'association d'un groupement stérolique actif avec une protéine.

D'autre part, Witschi souligne une différence notable entre les inducteurs tels que les révèlent, par exemple, les expériences de parabiose, et l'action des hormones sexuelles; ces dernières n'exerceraient que des effets stimulants, alors que les premiers manifestent principalement leurs effets inhibiteurs. Chaque inducteur produirait une substance +, stimulante, d'action assez localisée, et une substance −, inhibitrice, active à plus grande distance; les hormones agiraient en favorisant la sécrétion de la substance + (Witschi, 1942).

Il est certain que la première modification du free-martin bovin est l'inhibition du cortex; dans les expériences de parabiose ou de greffes orthotopiques chez les Batraciens, Witschi et Humphrey ont plusieurs fois souligné que l'action du mâle sur la femelle était surtout de nature inhibitrice, la masculinisation qui peut affecter la femelle apparaissant comme une réaction compensatrice. Aucun des stéroïdes essayés jusqu'à présent n'a provoqué la stérilisation et l'inhibition quasi-totale de l'ovaire comme on l'observe dans certains cas de parabiose ('free-martin effect').

Or, récemment Vannini (1945, 1946) a décrit des effets inhibiteurs produits par certains stéroïdes. Comme la testostérone, la progestérone masculinise les gonades des têtards de Grenouilles, et comme l'œstradiol, l'acétate de désoxycorticostérone est féminisant. En étudiant l'action de ces diverses substances Vannini reconnaît à chacune d'elles un mode d'action particulier. La testostérone favorise la médulla, la progestérone au contraire inhibe le cortex; dans les deux cas la gonade est masculinisée. L'œstradiol favorise le cortex, l'acétate de désoxycorticostérone inhibe la médulla, dans les deux cas la gonade est féminisée. La progestérone et l'acétate de désoxycorticostérone seraient capables d'agir dans la différenciation sexuelle comme les inducteurs embryonnaires, en provoquant une inhibition suivie d'une inversion sexuelle par compensation.

Dans une observation inédite, j'ai constaté que les trois fœtus mâles d'une Lapine injectée d'acétate de désoxycorticostérone entre le 5^e^ et le 15^e^ jour de gestation (20 mg. par jour, produit purissime Roussel) portaient en fin de gestation des testicules de taille réduite et qui paraissaient lésés; leur prostate est très peu développée et les organes génitaux externes ont une tendance hypospade. Ces modifications peuvent résulter d'une diminution de l'activité testiculaire; je soumets la question à une nouvelle étude. On sait que chez l'adulte l'acétate de désoxycorticostérone provoque une inhibition marquée des testicules (Courrier & Poumeau Delille, 1942).

D'autre part Wolff (1946, 1947) a comparé l'action de greffes de glandes génitales embryonnaires et celles des hormones sexuelles sur l'embryon de Poulet: il insiste sur l'identité des résultats. Les transformations des gonades, induites seulement dans le cas d'une greffe ovarienne sur un mâle, sont peu marquées, mais elles sont identiques à celles que l'on obtient dans certaines conditions sous l'action de l'œstradiol. Ces expériences ne révèlent pas de différence entre les hormones sexuelles et les inducteurs embryonnaires.

Est-on en droit, devant ce succès, d'assimiler les inducteurs embryonnaires aux hormones sexuelles ou à des stérols voisins? Il est indispensable auparavant de tenir compte de quelques difficultés, en particulier de certaines actions dites 'paradoxales', et de certains insuccès.

Parmi les actions paradoxales nous retiendrons celles qui sont en relation avec la dose d'hormone utilisée, parcequ'elles sont les plus caractérisées.* Le problème a été soulevé par Padoa (1936) qui constata que des têtards de *Rana esculenta* élevés dans de l'eau additionnée de folliculine étaient, contre toute attente, masculinisés

* Considérer comme 'paradoxal' le fait que l'androstérone (par exemple) exerce une action bisexuelle sur l'embryon, c'est me semble-t-il partir de l'idée préconçue que cette substance devrait être uniquement masculinisante, ce que dément l'expérience. Les potentialités de chaque hormone sont celles que lui révèlent les récepteurs. Bien entendu il n'est pas possible de considérer l'androstérone comme identique à l'inducteur sexuel masculin du Poulet.

et non féminisés. Gallien (1941) observe qu'une même préparation d'œstradiol est féminisante quand on l'administre en injections huileuses, et masculinisante lorsqu'on la donne dans l'eau de l'aquarium. Enfin Padoa (1942) élève des têtards de *R. esculenta* dans de l'eau contenant diverses concentrations d'œstradiol: la même substance est féminisante à faible dose (30 à 60γ/l.), masculinisante à dose élevée (250γ/l.).

D'autres actions paradoxales comparables ont été observées par Foote (1941), qui féminisa des Ambystomes par le propionate de testostérone, et par Crown (cité d'après Witschi, 1942), qui masculinisa des Xiphophores par l'œstrone.

Les actions paradoxales précédentes sont difficiles à comprendre si l'on admet que l'hormone représente par elle-même l'inducteur actif physiologiquement. Padoa (1942) propose l'interprétation suivante: la différenciation sexuelle des gonades de Grenouille serait dirigée par une seule substance identique aux hormones sexuelles ou très voisine, et dont la concentration plus ou moins élevée orienterait le sexe de la gonade dans un sens ou dans l'autre; le têtard qui produirait le plus de folliculine évoluerait dans le sens mâle. Cette hypothèse paraît difficilement compatible avec un grand nombre d'observations.*

Il convient de rapprocher des faits précédents d'autres constatations inattendues relevées chez les Batraciens. Dans le cas des parabiontes *R. sylvatica* ♀+*R. temporaria* ♀ les ovaires de la première femelle s'hypertrophient alors que ceux de la seconde subissent une régression. Après l'atrophie du cortex de *R. temporaria*, la médulla peut subir une croissance compensatrice et il en résulte une inversion partielle, dans une paire homosexuée (Witschi, 1932, Fig. 30). Dans les *Ambystoma maculatum* portant en greffe orthotopique une gonade du même sexe d'*A. tigrinum* (Humphrey, 1942*b*), ou dans des parabiontes *Ambystoma*+*Triturus* (Witschi & McCurdy, 1943), les ovaires de l'une des espèces (*A. maculatum* ou *Triturus*) sont inhibés par les ovaires de l'autre espèce, il en est de même des testicules. Ces inhibitions sont moins marquées généralement que celles qui s'observent dans les paires hétérosexuées et sont indépendantes de la présence ou de l'absence de l'hypophyse (Humphrey, 1942*b*). Humphrey pense que dans l'organisme les gonades exercent l'une sur l'autre une inhibition de croissance réciproque; l'action de la grosse gonade greffée s'expliquerait par l'intensification de cette inhibition due sans doute à la grande taille du greffon. Witschi suppose qu'il existe dans tout organisme des substances de croissance, 'ovarial and testicular growth substances', différentes des hormones sexuelles ou hypophysaires et nécessaires à la croissance des gonades. Lorsque l'un des parabiontes utilise toute la quantité disponible de cette substance, il n'y en a plus assez pour son partenaire. Le mécanisme de cette inhibition serait donc, selon Witschi, différent de celle produite par les inducteurs sexuels de la gonade; il ne s'agirait pas là d'un autre type d'action paradoxale.

Nous rapellerons enfin les insuccès obtenus chez les Mammifères chez lequels on n'a jamais réussi à inverser le sexe de la glande génitale sous l'action des hormones sexuelles. Plusieurs méthodes ont été mises à l'épreuve: (1) Buyse (1935) a greffé des ébauches encore indifférenciées de gonades sous la capsule rénale de Rats adultes. La reprise des greffons est excellente; sur 246 greffes il se différencie 60% de testicules, 18% d'ovaires et 22% d'ovotestis ou de glandes de sexe indéterminé; la répartition de ces divers types est sans rapport avec le sexe de l'hôte. Les hormones de l'hôte n'ont pas altéré le sens de la différenciation sexuelle des greffons et ne sont pas responsables de la transformation en ovotestis de certains ovaires (*v.* aussi Moore & Price, 1942). (2) Dantchakoff (1936, 1937*a*, *b*) injecte du propionate de testostérone directement dans les annexes du fœtus de Cobaye *in utero.* Après avoir décrit en 1939 des femelles dont les ovaires avaient pris des caractères d'ovotestis, l'auteur est revenu sur cette interprétation et fait remarquer que la testostérone 'est impuissante à inciter des transformations dans la gonade femelle' (Dantchakoff, 1947). (3) La testostérone ou l'œstradiol administrés directement au petit Opossum dès le début de sa différenciation sexuelle n'altèrent pas le sexe des glandes génitales (Burns, 1939*a*, *b*, 1942; Moore, 1939, 1941). On ne peut songer à expliquer cet insuccès par les difficultés d'administration de l'hormone comme dans le cas des Placentaires. (4) A la suite de Greene & Ivy (1937) on a obtenu l'intersexualité de l'embryon de divers Placentaires, en injectant des hormones à la mère en gestation (cf. Greene *et al.* 1939*a*, 1940; Raynaud, 1942; Turner, 1939). La structure des gonades reste inchangée; on peut évidemment se demander si l'hormone parvient à l'embryon suffisamment tôt, et en quantité suffisante. Dans la plupart des espèces le propionate de testostérone ou l'œstradiol sont des abortifs d'autant plus puissants que la grossesse est moins avancée. Les expériences ont donc été réalisées en injectant les femelles au cours de la deuxième moitié de la gestation. J'ai pu tourner cette difficulté en utilisant des androgènes doués de propriétés progestatives, la prégnèninolone et la méthyltestostérone. Ces deux dérivés de la testostérone masculinisent les gonades de *Rana temporaria* (Jost, 1943, 1945); ils peuvent en outre remplacer les corps jaunes gravidiques chez la femelle ovariectomisée, comme la progestérone. J'ai pu administrer en une série d'injections régulièrement espacées, plusieurs centaines de mg. de ces androgènes (dans un cas 1·3 g.) à des Lapines pleines, en commençant le traitement au moment où les œufs étaient au stade morula, ou blastocyste: ce traitement a laissé les ovaires fœtaux sensiblement normaux (prégnèninolone) ou a provoqué une certaine hypertrophie de la médulla (méthyltestostérone) qui a gardé un aspect ovarien (Jost, 1947*f*).

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* Lepori (1946) fait intervenir un relais hypophysaire. Son interprétation nécessite plusieurs hypothèses non encore confirmées par l'expérience.

L'androgène traverse-t-il le placenta et parvient-t-il à l'embryon dès les stades les plus précoces? On ne peut encore répondre d'une manière certaine. En tout cas l'hormone agit sur le fœtus de Lapin avant le 22^e^ jour puisqu'elle provoque dès ce moment le bourgeonnement prostatique des femelles, mais elle n'empêche pas le développement des cordons corticaux ovariens qui se produit à la même date. Dans les free-martins, la poussée de ces cordons, ou le cortex déjà constitué, sont au contraire inhibés, mais aucun bourgeon prostatique ne s'édifie (stade entre 5·1 et 7·5 cm., Bissonnette, 1928*b*).

En ce qui concerne donc l'action sur les glandes génitales de l'embryon des Mammifères, on ne peut encore assimiler aucun des stéroïdes essayés jusqu'à présent au facteur actif dans l'embryon mâle des Bovidés.

(2) *Différenciation sexuelle somatique*

L'intervention de sécrétions génitales dans la différenciation sexuelle somatique est bien établie à présent. Seul le cas de l'Opossum est encore douteux et nécessite de nouvelles recherches.

Les sécrétions des gonades jouent-elles un rôle dans la 'mise en place' des gonoductes, qui précède leur évolution dans le sens mâle ou femelle? Le canal de Wolff s'édifie longtemps avant les gonades, la question se pose donc seulement pour les canaux de Müller.

Chez les Batraciens cette première phase est indépendante des gonades ('self-differentiating period'); elle est suivie par la 'sex-controlled period', qui débute une fois les canaux mis en place (Witschi, 1929; Gallien, 1944). Chez les Mammifères le canal de Müller du mâle se développe généralement comme celui de la femelle, puis régresse lorsqu'il a atteint sa complète extension; parfois il est plus réduit que celui de la femelle à des stades précoces—c'est le cas du Veau (Bissonnette, 1924) ou de l'Opossum. Ces différences sont peut-être en rapport avec une sécrétion testiculaire précoce.

Selon Witschi (1942) il existe un 'tube inductor' situé à l'extrémité en croissance de l'oviducte et responsable de sa différenciation et de sa poussée longitudinale. Dans certaines conditions les hormones sexuelles peuvent supprimer ou arrêter la formation du conduit (Foote, 1941, chez les Batraciens; Gaarenstroom, 1939; Stoll, 1945, chez le Poulet); dans ces cas les hormones détruiraient l'inducteur tubaire tout en étant capables d'hypertrophier le segment déjà constitué (Witschi, 1942). Il faut distinguer cette inhibition de la formation du canal, qui n'est pas un processus normal de l'ontogenèse, de sa régression telle qu'elle se produit chez le mâle pendant la différenciation sexuelle et après se mise en place normale. Boss & Witschi (1947) considèrent ce mode d'action particulier des hormones comme une maturation précoce de l'ébauche, supprimant sa capacité de développement.

(*a*) *Mode d'action des sécrétions génitales.* On a fait plusieurs hypothèses pour expliquer les modalités précises de la différenciation des gonoductes, surtout en ce qui concerne les Mammifères. Dans ce groupe l'histogenèse génitale somatique est la plus complexe; chez le Poulet, par exemple, le canal de Wolff n'est pas un caractère sexuel durant toute la vie embryonnaire et il n'existe pas de glande annexe.

Wiesner (1934, 1935), à la suite d'expériences sur des Rats nouveaux-nés, a admis que seule l'édification du tractus génital mâle nécessiterait une sécrétion gonadique (testiculaire), alors que le tractus génital féminin pourrait se constituer sans l'intervention d'aucune hormone.

A la suite des expériences de Greene *et al.* (1938*b*, 1940) et de Raynaud (1939, 1942) révélant le pouvoir féminisant des œstrogènes sur l'embryon des Mammifères, on préféra une théorie 'dihormonale' à cette conception 'mon-hormonale'. Ainsi Raynaud (1942), par exemple, a supposé que chacun des deux sexes produit une substance sexuelle responsable du développement des gonoductes homologues, l'involution des voies hétérologues étant due à l'absence de la sécrétion correspondante. Selon cette manière de voir un embryon castré ne posséderait aucune voie génitale.

Les expériences de castration de l'embryon permettent de donner une base expérimentale à ce problème. On constat que dans des embryons mâles de Lapin castrés avant ou peu après le début de la différenciation sexuelle somatique les canaux de Wolff régressent complètement, mais les canaux de Müller persistent et constituent des organes dont la conformation rappelle celles des organes de la femelle. Dans les femelles castrées les canaux de Müller persistent seuls également.

On est donc conduit à admettre que, quel que soit le sexe génétique de l'embryon, le canal de Müller évolue s'il n'est pas *inhibé* par le testicule embryonnaire, le canal de Wolff involue, comme le corps de Wolff, s'il n'est pas *stabilisé* par ce même testicule.

Le testicule joue donc un rôle prépondérant dans l'apparition des différences qui distinguent les deux sexes, d'autant plus qu'il tient également sous son contrôle le développement de la prostate et la morphologie des organes génitaux externes. Cette conclusion ne signifie pas que l'ovaire ne joue aucun rôle dans la différenciation sexuelle. Nous avons en effet déjà noté que dans des embryons femelles de Lapins castrés peu après le début de la différenciation sexuelle, les organes müllériens avaient un diamètre plus faible que dans les femelles normales. L'ovaire semble donc leur donner une stimulation précoce. En outre, il n'est pas impossible que les ovaires produisent une sécrétion capable d'inhiber les canaux de Wolff, bien que ceux-ci régressent en leur absence; il pourrait exister une double assurance.

Par ailleurs diverses observations semblent montrer qu'il faut davantage de sécrétion testiculaire pour inhiber les canaux de Müller que pour assurer la persistance des canaux de Wolff. Cette remarque paraît permettre une interprétation assez simple de certaines malformations du tractus génital humain caractérisées par la persistance de dérivés müllériens à côté des voies mâles: le testicule embryonnaire a peut-être souffert d'une légère déficience.

Les sécrétions génitales de l'embryon passent-elles dans la circulation générale, ou bien agissent-elles d'une manière très localisée? Des expériences de parabiose sur l'embryon de Lapin (Jost, 1946*a*) n'ont révélé aucune action de l'un des sexes sur l'autre, mais elles étaient faites à un stade tardif (23 jours). Il faudra répéter ces essais sur des embryons plus jeunes. Les résultats de certaines castrations unilatérales donnent quelques indications. Si le testicule unique provoque un développement normal de la prostate et des organes génitaux externes, il n'agit parfois qu'assez faiblement sur les gonoductes situés du côté castré, surtout lorsque la castration unilatérale a été effectuée assez tôt, et que le testicule unique est mal développé; or, les organes génitaux externes sont situés à une distance plus grande du testicule que le canal déférent opposé. L'action relativement unilatérale du testicule embryonnaire s'explique peut-être par la disposition de la vascularisation?

Enfin l'action des sécrétions des gonades sur les voies génitales présente des particularités qui s'expliquent sans doute par la nature du récepteur, le territoire embryonnaire, qui après une phase de réceptivité subit une détermination irréversible. Les sécrétions génitales embryonnaires n'ont pas besoin d'agir durant toute la durée de l'organogenèse d'une ébauche pour que celle-ci puisse poursuivre son développement (*v.* p. 212). La sécrétion embryonnaire semble marquer les ébauches d'une détermination irréversible à partir d'une certaine date. Ainsi le canal déférent régresse lorsque la castration est antérieure à 24 jours, mais persiste lorsque l'embryon est castré plus âgé ou après la naissance.

Ces observations rejoignent celles faites par Wolff (1938) au sujet de l'action des hormones sexuelles sur les gonoductes de l'embryon de Poulet. L'oviducte pour être d'une manière définitive 'stabilisé' par la folliculine ou 'inhibé' par l'androstérone n'a 'besoin que d'une "impulsion" qui doit s'exercer en temps utile et qui représente un processus unique en cours du développement'; la phase de réceptivité est très précoce (cf. Et. & Em. Wolff, 1947) et précède de beaucoup les manifestations morphologiques. Ces particularités rapprochent l'action des facteurs embryonnaires actifs sur les voies génitales, des actions inductrices observées aux premiers stades de l'embryogenèse; elles s'opposent à la réversibilité des modifications subies par les gonades.

(*b*) *Nature des sécrétions génitales.* On envisagera ici ce problème en posant les deux questions suivantes: les sécrétions embryonnaires qui président au modelage du soma, sont-elles identiques aux substances inductrices des gonades, ou aux hormones de l'adulte?

(1) Selon Witschi la cortexine et la médullarine n'ont aucun rôle dans la différenciation sexuelle somatique. Il interprète les free-martins en supposant que la médullarine du mâle agit uniquement sur les ovaires de la femelle. La modification dans le sens testiculaire de ces ovaires est responsable des transformations des voies génitales; il y a une relation entre le degré de développement de la médulla et le degré de masculinisation du tractus somatique alors qu'il n'y a pas de relation directe entre le volume sanguin échangé par les fœtus et le taux de masculinisation (cf. Witschi, 1939).

Bissonnette (1928*b*) pensait que l'hormone provenant du mâle pouvait en même temps inhiber un cortex déjà constitué et les canaux de Müller, ces deux modifications sont d'en effet concomitantes.

Et. & Em. Wolff (1947) ont récemment apporté des faits qui semblent parler en faveur de l'identité des facteurs actifs sur les gonades et les voies génitales. Chez l'embryon de Poulet mâle les canaux de Müller régressent vers le 12ᵉ jour d'incubation; or leur stabilisation ou leur inhibition est déterminée longtemps à l'avance. Pour en provoquer la stabilisation par la folliculine il faut agir avant le 6ᵉ jour; lorsque la folliculine est donnée après le 6ᵉ jour, la gonade peut être féminisée mais le canal de Müller ne persiste pas. Si la détermination est aussi précoce au cours du développement normal, elle est donc le fait de la sécrétion active avant le 6ᵉ jour, c'est à dire des inducteurs sexuels de la gonade. On compléterait heureusement ces recherches en réalisant la castration de l'embryon de Poulet avant ou après le 6^e^ jour.

(2) La différenciation du tractus génital somatique fœtal est-elle sous le contrôle des mêmes hormones sexuelles qui dirigent les caractères sexuels secondaires post-pubéraux? Dans le cas des Batraciens, Gallien (1944) admet cette identité. Les nombreuses expériences d'intersexualité réalisées chez l'embryon apportent d'importantes données dans l'étude de ce problème. Mais il est utile de faire les deux remarques suivantes au sujet des résultats obtenus.

(i) Avant mes expériences sur l'embryon de Lapin, on n'a jamais étudié l'action des hormones sexuelles sur la différenciation sexuelle de fœtus castrés. Or la sécrétion des gonades fœtales peut venir interférer avec l'hormone étrangère. Je citerai deux exemples: (*a*) Greene *et al.* (1942) observent des différences légères dans la réponse des deux sexes d'embryons de Rat à un même traitement hormonal, et supposent que ces différences s'expliquent par l'intervention des sécrétions génitales embryonnaires. (*b*) L'interprétation des effets de l'œstradiol sur l'embryon mâle de Rat (Greene *et al.* 1940) ou de Souris (Raynaud, 1942) peut être envisagée de deux manières: la persistance des canaux de Müller, la disparition des canaux de Wolff et l'absence de la prostate, par exemple, peuvent se concevoir comme un effet direct de l'hormone sur ces récepteurs, ou comme un effet indirect résultant de la suppression de la sécrétion testiculaire.

(ii) Parmi les 'actions paradoxales' ou 'bisexuelles' produites par les hormones sexuelles je pense qu'il faut distinguer les réactions 'organo-génétiques' responsables du développement ou de la régression de certains organes, des réactions que l'on peut qualifier de 'fonctionnelles' et qui se manifestent par une hypertrophie ou une stimulation de croissance d'organes normalement présents dans l'embryon. Je préciserai à l'aide d'un exemple: le propionate de testostérone exerce une action progesto-mimétique sur l'utérus de divers Mammifères adultes, en particulier la Chatte; or injectée à la Chatte pleine en fin de gestation, cette substance entraîne l'hypertrophie utérine des embryons femelles (Courrier & Gros, 1938). J'interpréte cette observation en admettant que dès la fin de la vie embryonnaire l'utérus de la Chatte répond à cette hormone comme il le fait chez l'adulte, ce n'est pas là une réaction 'paradoxale' au point de vue embryologique.* Il est possible que la même explication puisse s'appliquer aux Batraciens, puisque l'hormone mâle provoque l'hypertrophie de l'oviducte des adultes (Wolf, 1939) et des jeunes individus (Foote, 1941). Chez certains Oiseaux adultes castrés le propionate de testostérone hypertrophie les oviductes (Witschi, 1943). En ce qui concerne l'Opossum, Burns (1945) a récemment insisté sur l'action bisexuelle des fortes doses de propionate de testostérone, qui déterminent la présence dans les deux sexes de canaux de Müller hypertrophiés à côté des canaux de Wolff. S'agit-il seulement d'une hypertrophie de restes müllériens qui persistent normalement à cet âge chez le mâle, ou bien l'androgène a-t-il provoqué la persistance d'organes qui normalement disparaissent?

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* Certains territoires répondent parfois à une date extraordinairement précoce: la prégnèninolone peut faire apparaître les callosités de *Discoglossus pictus* avant que les membres antérieurs ne soient extériorisés (Gallien, 1945).

D'autres réactions paradoxales ont été observées sous l'action des œstrogènes: stimulation des canaux de Wolff de la Grenouille (Gallien, 1944), de l'Opossum (Moore, 1939) et de l'embryon femelle de Rat (Greene *et al.* 1939*b*), ou de Souris (Raynaud, 1942); léger développement prostatique de l'embryon de Rat ou de Souris. Ces dernières réactions sont d'autant plus curieuses que chez le Rat et la Souris mâle l'œstradiol inhibe en grande partie les canaux de Wolff et la prostate, si bien que le tractus génital finit par présenter certaines ressemblances dans les deux sexes (Greene *et al.* 1939*b*). Peut-être la comparaison entre les réponses à un même traitement, et après gonadectomie, d'embryons indifférenciés, d'embryons ou de nouveaux-nés dont l'organogenèse génitale est terminée, et d'adultes, permettra-t-elle de minimiser l''incohérence' apparente de ces réactions. Il n'en reste pas moins vrai que ces réactions paradoxales posent encore un grave problème si l'on admet l'identité des agents morphogènes embryonnaires et des hormones de l'adulte.

Après ces remarques il est nécessaire de souligner l'inefficacité du traitement hormonal dans certains cas. Le propionate de testostérone est capable d'inhiber les canaux de Müller chez les Batraciens (Foote, 1941; Gallien, 1944) et chez les Oiseaux (Willier *et al.* 1938). Mais Foote constate que la testostérone a empêché les canaux de Müller de se former et non provoqué leur régression, comme le fait normalement le testicule (*v.* p. 223); ce point semble n'avoir pas été complètement précisé par les autres auteurs cités ici. L'hormone mâle ne provoque l'inhibition müllérienne ni chez l'Opossum ni chez les Placentaires (Greene *et al.* 1938, par exemple, ne signalent l'interruption des voies müllériennes que dans un petit nombre de cas—2 sur 69—jugés anormaux).* Chez les Mammifères les androgènes possèdent le même pouvoir stimulant que le testicule embryonnaire (développement des canaux de Wolff, de la prostate, des organes génitaux externes), mais non le même pouvoir inhibiteur sur les canaux de Müller. Ce problème mérite d'être étudié de près, et la seule façon valable de l'aborder est d'opérer sur des fœtus castrés. Dans une première série d'expériences sur ce sujet j'ai noté (Jost, 1947*c*, *f*) que dans des mâles castrés ayant reçu en implantation un cristal de propionate de testostérone les organes masculins se sont développés, mais les canaux de Müller n'ont pas été inhibés d'une manière complète, même lorsque la prostate paraissait plus développée que normalement. De nouvelles recherches sont nécessaires, mais il était bon de signaler que les expériences réalisées jusqu'à présent laissent subsister une différence importante entre l'action de la sécrétion du testicule embryonnaire et celle que l'on a pu reconnaître aux androgènes de synthèse.

Je voudrai enfin tout au moins évoquer l'important problème de la 'neutralité' du soma. En l'absence de toute stimulation hormonale, les potentialités des somas des deux sexes sont-elles rigoureusement les mêmes? La question a encore été récemment examinée par Burns (1942) au sujet de l'Opossum. Cet auteur constate que sous l'influence d'un même traitement à l'hormone mâle la prostate du mâle est toujours plus développée que celle de la femelle. Dans le développement de toute ébauche il interviendrait, selon Burns, en plus du contrôle humoral, des facteurs internes liés au sexe génétique, qui détermineraient la spécificité de la réaction et la capacité de croissance de l'ébauche. Les faits cités par Burns ne seraient cependant tout à fait convaincants que s'ils avaient été établis sur des embryons castrés, les sécrétions génitales pouvant interférer avec les hormones injectées. J'ai déjà signalé l'opinion d'Ancel (1946) et les expériences de Padoa (1947), selon lesquelles les deux sexes seraient inégalement sensibles à une même hormone (*v.* p. 218).

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* Par contre, Mombaerts (1944) signale la disparition de plusieurs tronçons müllériens dans les femelles de Hérissons intersexuées sous l'action de la testostérone (4 cas). Chez la Souris (Raynaud, 1942) et le Lapin (Jost, 1947*f*) l'androgène peut inhiber la fusion de la partie postérieure des deux canaux.

Les facteurs génétiques du sexe jouent peut-être dans certains cas un rôle important, il appartiendra à l'expérience de les mettre en évidence avec précision sur des ébauches qui n'aient pas été soumises au préalable à l'action des sécrétions des gonades fœtales.

V. CONCLUSIONS

L'intervention d'un contrôle humoral de la différenciation sexuelle est bien établie, et des faits anciens ou nouveaux permettent d'écarter certaines objections présentées par Moore (1944) à la 'théorie hormonale' de la différenciation du sexe. Diverses observations ont même permis de préciser certaines particularités du mode d'action des sécrétions embryonnaires.

Mais aucun fait n'est encore venu établir d'une manière définitive la nature de ces sécrétions. Deux points de vue sont défendus. Pour Witschi, et d'autres biologistes après lui, les inducteurs sexuels de la gonade (cortexine et médullarine) sont absolument différents des hormones sexuelles qui tiennent sous leur dépendance tous les caractères sexuels somatiques. Cependant, en ce qui concerne les Oiseaux et les Mammifères, on ne peut pas encore tenir pour démontré le fait que les substances inductrices de la gonade ne sont pas également les agents de la différenciation sexuelle des voies génitales de l'embryon. Selon d'autres auteurs, en particulier Wolff, il n'y a pas de raison d'établir de différence entre les sécrétions génitales, depuis les stades embryonnaires jusqu'à l'adulte. Mais l'examen des faits expérimentaux ne paraît pas permettre d'assimiler aux sécrétions des gonades embryonnaires aucun des stéroïdes dont l'action a été étudiée sur l'embryon jusqu'à présent. *A priori*, et en songeant surtout aux Mammifères et aux Oiseaux dont toute l'histogenèse génitale a lieu durant la vie embryonnaire, il ne serait pas impossible d'envisager une troisième hypothèse selon laquelle la différenciation sexuelle germinale et somatique de l'embryon relèverait des mêmes sécrétions embryonnaires distinctes des hormones sexuelles de l'adulte.

Ces incertitudes ou ces hypothèses divergentes stimulent les recherches et suscitent de nouvelles expériences qui font de ce problème à l'étude depuis plus de trente ans une question de pleine actualité.

VI. RÉSUMÉ

1. Le contrôle hormonal de la différenciation du sexe admis par Bouin & Ancel en 1903 a trouvé une belle confirmation dans l'observation et l'interprétation des free-martins (Lillie, 1916; Keller & Tandler, 1916).

2. Les greffes orthotopiques effectuées par Humphrey sur l'Ambystome et les greffes intracœlomiques de Wolff sur l'embryon de Poulet établissent la production de sécrétions morphogènes par les gonades embryonnaires; Jost a également obtenu un cas positif chez l'embryon de Lapin.

3. La parabiose entre larves indifférenciées de Batraciens montre aussi que les gonades des deux sexes produisent durant leur différenciation sexuelle des substances capables d'agir sur les gonades du sexe opposé (Burns, Humphrey, Witschi).

4. La castration effectuée avant le début ou pendant la différenciation sexuelle somatique montre que celle-ci obéit à un contrôle humoral, chez le Triton (de Beaumont) et chez le Lapin (Jost). Les résultats négatifs de Moore sur l'Opossum sont discutés; ils s'expliquent peut-être par la date trop tardive de la castration; chez le fœtus de Lapin, pour supprimer tout développement prostatique, par exemple, la castration doit en effet intervenir avant l'apparition des premiers bourgeons prostatiques.

5. L'extraction chimique d'hormones des gonades fœtales a pu être réalisée chez le Veau, mais n'apporte encore que des données fragmentaires.

6. On peut voir un argument parlant en faveur de la production d'hormones par la gonade fœtale dans l'action des hormones sexuelles exogènes sur les stades embryonnaires.

7. D'après les expériences réalisées jusqu'à présent, ni l'hypophyse, ni les surrénales, ni les thyroïdes ne semblent jouer de rôle sensible dans la différenciation sexuelle.

8. La différenciation sexuelle des gonades commence par celle de leurs constituants somatiques qui libèrent des inducteurs corticaux et médullaires. C'est la prédominance de l'un de ces inducteurs qui oriente l'évolution de la gonade dans le sens femelle ou mâle (Witschi).

9. La nature des substances inductrices de la gonade fait l'objet de points de vue opposés. Pour Witschi elles seraient entièrement différentes des hormones sexuelles de l'adulte. Pour d'autres auteurs elles seraient soit identiques (Dantchakoff) soit de même nature (Wolff) que les hormones sexuelles. Les faits expérimentaux ne permettent pas encore d'apporter une conclusion certaine.

10. D'après les expériences de castration de Jost le testicule fœtal joue un rôle essentiel dans la différenciation sexuelle somatique des Mammifères. Le testicule provoque le développement des glandes prostatiques, des organes génitaux externes masculins et des canaux de Wolff; il inhibe les canaux de Müller. Dans les castrats des deux sexes, et dans les femelles normales, les canaux de Wolff régressent et les canaux de Müller persistent. Ces résultats, très voisins de ceux que postulait Wiesner, ne prouvent cependant pas que l'ovaire fœtal ne produit aucune sécrétion.

11. Il n'est pas encore possible de dire si les sécrétions génitales qui dirigent la différenciation sexuelle somatique sont identiques soit aux inducteurs sexuels de la gonade, soit aux hormones de l'adulte.

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ADDENDUM

Des travaux récents ont été consacrés à certains des problèmes soulevés dans cet article; je les cite en indiquant quel paragraphe ils complètent.

II (3). C. R. Moore a réuni dans un volume (*Embryonic Sex Hormones and Sexual Differentiation*, 81 pp., C. C. Thomas éd., Springfield, Ill., 1947) les faits qui sont à l'appui de sa conception anhormonale de la différenciation sexuelle du tractus génital.

Par ailleurs, A. Raynaud et M. Frilley ont récemment apporté des expériences qui confirment certains de mes résultats concernant la castration précoce ('Effets sur le développement du tractus génital des embryons de Souris, de la destruction des ébauches de leurs glandes génitales par une irradiation au moyen des rayons X, à l'âge de 13 jours', *C.R. Soc. Biol., Paris*, 1947, **141**, 1134). Il est intéressant d'appliquer à l'étude de ce problème des méthodes convergentes, cependant la technique des irradiations par les rayons X, utilisée par ces auteurs pour détruire les glandes génitales, est moins favorable que la méthode chirurgicale, car elle ne conduit qu'à une destruction incomplète des gonades et entraine de plus une destruction presque complète des gonoductes. Dans les mâles aux testicules lésés, la partie postérieure du tractus génital, conservée après l'intervention, subit une inversion de la différenciation sexuelle dans le sens femelle.

II (4). P. Leroy ('Effet androgène d'extraits embryonnaires de Poulet sur la crête du Chapon', *C.R. Acad. Sci., Paris*, 1948, **226**, 520) a extrait de l'embryon de poulet entre 14 et 19 jours, une préparation active sur la crête du chapon. Il faut au moins 20 embryons pour obtenir une action. Dans le même laboratoire (M. Caridroit), Riboulleau avait déjà décelé des œstrogènes dans les œufs.

III. En rédigeant les lignes relatives au rôle de l'hypophyse dans le développement du tractus génital, je n'avais tenu compte que des résultats publiés jusque là. Des expériences personnelles récentes effectuées sur le Lapin, obligent à poser à nouveau la question. Dans les embryons mâles décapités à 19 jours, c'est à dire avant le début de la différenciation sexuelle somatique, et étudiés peu avant la naissance (28 j), on relève des signes d'insuffisance testiculaire très nets et d'autant plus marqués que le récepteur est plus éloigné des testicules (prostate comparable à celle des embryons castrés à 21 jours; organes génitaux externes de type féminin comme dans les castrats) (A. Jost, 'Influence de la décapitation sur le développement du tractus génital et des surrénales de l'embryon de Lapin', *C.R. Soc. Biol., Paris*, 1948, **142**, sous presse).

Dans un foetus décapité au même âge et traité par de l'hormone gonadotrope (P. M. S. Roussel) ces signes d'insuffisance testiculaire n'existent pas. Je poursuis ces expériences pour voir si l'influence de la décapitation est bien due, comme je le suppose, à l'absence de l'hypophyse. Il sera intéressant de réaliser des greffes d'hypophyse fœtale sur les fœtus décapités.

IV. Dans un important travail, B. Mintz a étudié en détail l'action du propionate de testostérone sur des larves d'Ambystome de constitution génétique femelle bien connue ('Effects of testosterone propionate on sex development in female Ambystoma larvae', *Physiol. Zool.* 1947, **20**, 355). Elle constate en particulier que si certains territoires sont masculinisés (cloaque), les ovaires ne subissent pas d'inversion dans le sens mâle, ce qui pourrait s'expliquer par le fait que l'androgène lèse profondément le blastème mésonéphrétique nécessaire à la constitution de la partie médullaire de la gonade; alors même que le cortex est très réduit, la gonade n'est pas inversée. Chez les Urodèles le propionate de testostérone aurait une action fortement gynogène (v. aussi Foote, 1941).

Poursuivant la comparaison expérimentale des sécrétions du testicule fœtal et du testicule adulte, j'ai récemment greffé des testicules de fœtus de Rats de 15 ou 16 jours sur la vésicule séminale de Rats adultes castrés (A. Jost, 'Activité androgène du testicule fœtal de Rat greffé sur l'adulte castré', *C.R. Soc. Biol., Paris*, 1948, **142**, sous presse). Les hôtes sont sacrifiés lorsque le tissus fœtal atteint 21 jours (date approximative de la naissance). Dans ces conditions, en 5 ou 6 jours, la greffe testiculaire fœtale exerce sur le caractère sexuel de l'adulte une activité androgène intense mais localisée. Il faut naturellement penser à l'activité de l'hypophyse de castration de l'hôte, et se demander si l'interstitielle fœtale a pu subir une maturation précoce. Pour répondre à cette question il est nécessaire de rechercher: 1^e si dans l'organisme fœtal du Rat l'hypophyse stimule normalement le testicule; 2^e) si en greffe sur des adultes castrés et hypophysectomisés le testicule fœtal jouit encore de la même activité androgène.

Notons enfin que des greffes de surrénales fœtales, réalisées dans les mêmes conditions, sont restées sans action décelable, ce qui est en accord avec les faits exposée dans le chapitre III.

ERRATA

Biol. Rev., vol. 23, no. 2

P. 203, l. 6: omettre 'nous'.

P. 206, l. 5 d'en bas: pour '*tigrinum* ♀', lire '*tigrinum* ♂'.

P. 209, l. 2: lire '(A) d'un mâle... (B) d'une femelle'.

P. 212, ll. 6, 5 et 4 d'en bas: transposer le mot 'suivante' et la phrase 'et se developpe dans l'abdomen'. Pour 'de permet', lire 'ne permet'.

CIRCULATORY CYCLES IN THE VERTEBRATES[1]

By H. W. MOSSMAN

Department of Anatomy, University of Wisconsin, Madison, U.S.A.

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I. INTRODUCTION

A few years ago Prof. C. L. Turner called my attention to the fact that the blood supply of the yolk sac of teleost fishes comes from somatic veins, such as the caudal and cardinals, instead of from vitelline arteries branching off from the aorta as in amniotes. Since then, over a period of years, in trying to make a comprehensive survey of the nature of the foetal membranes of all vertebrate groups, I have become more and more impressed with the possible significance of this and of a number of other differences and similarities in the sequence with which the blood reaches the various regions and organs of the body, both in the adults and developing young of the different groups of vertebrates. There are, I believe, a considerable number of important ontogenetic, phylogenetic, and physiological implications in the long known facts concerning the comparative anatomy and embryology of the circulatory system which have not been recognized or have been forgotten. This attempt to summarize some of the more pertinent information, and to call attention to some of its implications, has been undertaken in the hope that others might find challenging problems in this field as well as useful tools in the form of experimental animals for the further exploitation of problems already being investigated.

To avoid repetition, no further introductory discussion of the many interesting problems, such as those connected with the blood supply of the yolk sac, will be undertaken. The features of the comparative anatomy and embryology will be outlined first, followed by a discussion of the ontogenetic, phylogenetic, and physiological implications. The Cyclostomata are not included, but one useful reference is given in case some reader is interested in this group (Daniel, 1934).

II. COMPARATIVE ANATOMY AND EMBRYOLOGY

Yolk sac. The primary circulatory cycle is fundamentally the same in all vertebrate (craniate) embryos (Fig. 1, stage 1). The heart pumps the blood through the ventral aorta and first aortic arch to the dorsal aorta from which it flows into

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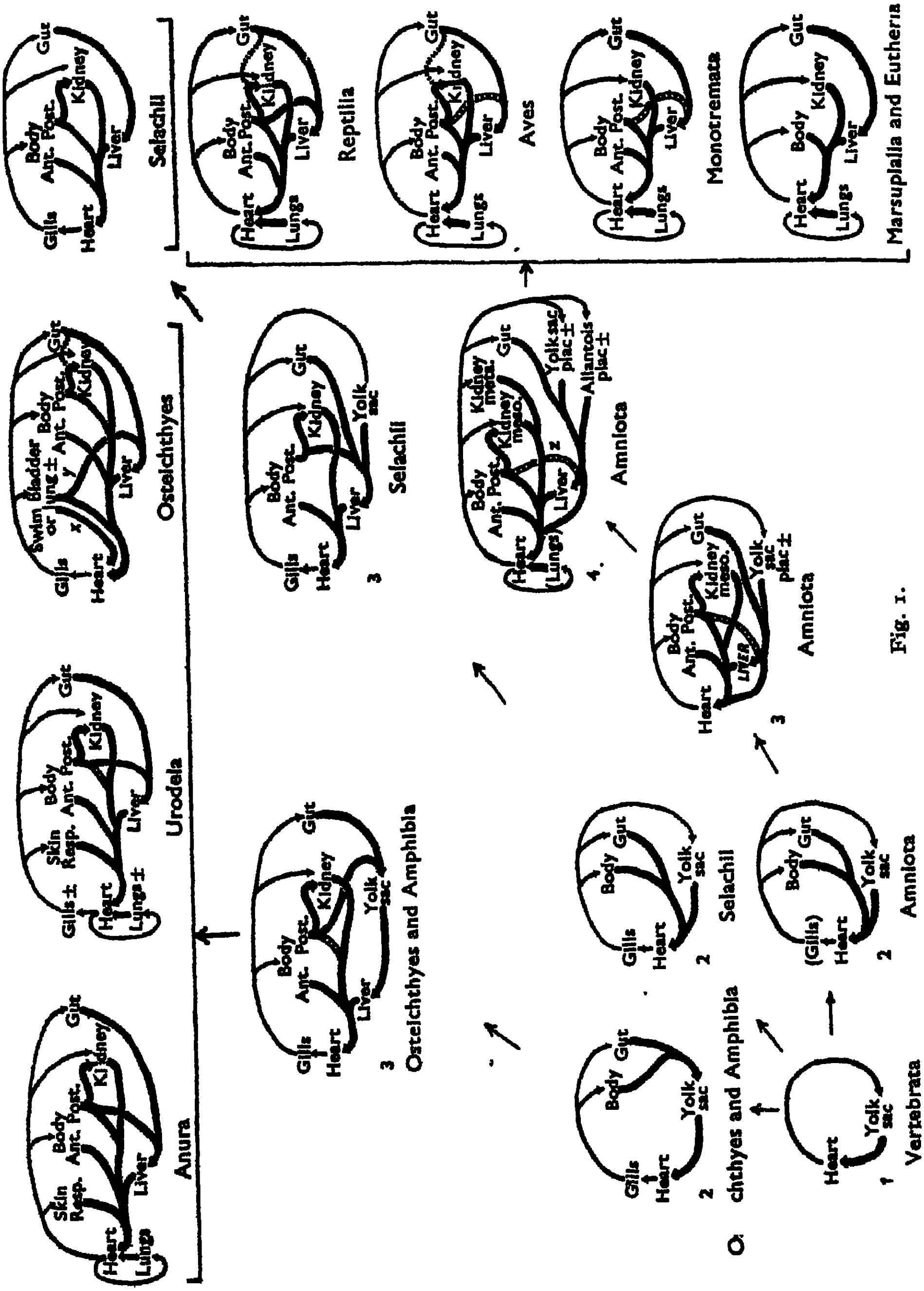
Vertebrata
Heart
Yolk sac
1
Osteichthyes and Amphibia
Gills
Heart
Body
Gut
2
Selachii
Amniota
(Gills)
3
4
Liver
Kidney
Kidney meso.
Kidney meta.
Ant. Post.
Yolk sac plac ±
Allantois plac ±
Lungs
Swim bladder or lung ±
x
y
z
Anura
Urodela
Osteichthyes
Reptilia
Aves
Monotremata
Marsupialia and Eutheria
Skin Resp.
Gills ±
Lungs ±

Fig. 1.

the capillary net on the yolk sac. This net then drains into the venous end of the heart to complete the cycle.

As soon as branches from the aorta to the body structures are established, a marked difference rapidly develops between the circulatory cycle sequence in the Osteichthyes (bony fishes) and Amphibia* on the one hand, and the Selachii and Amniota on the other (Fig. 1, stage 2). In the bony fishes and amphibians (Ryder, 1882, 1884, 1885; Wenckebach, 1886; Hochstetter, 1888; Anthony, 1918; Grodziński, 1924, 1925; Kryžanovsky, 1934) the body capillary system becomes interposed between the aorta and the yolk sac in such a manner that all the blood reaching the yolk sac must first pass through a capillary bed in the body. The vessels actually feeding the yolk sac plexus then are the posterior cardinal and the caudal veins, and often the subintestinal and common cardinals. In the Selachii and Amniota direct arterial branches to the yolk sac are maintained, and the venous blood from the body is carried directly to the sinus venosus by the cardinal system, by-passing the yolk sac completely.

It should be understood here that the so-called yolk sac of Osteichthyes, Amphibia, and Selachii is of the trilaminar type; that is, it is composed of ectoderm, mesoderm, and endoderm, and so is in a sense a modification of the body wall and gut wall not yet separated by coelomic spaces. Eventually coelomic spaces do develop between the somatopleure and splanchnopleure of the still yolk-laden sac of the Selachii, but this apparently does not happen in most Osteichthyes and Amphibia. Furthermore, in the fishes, and possibly to some extent in amphibians, but never in the selachians, the parietal pericardium is often an important part of the so-called yolk sac, and is covered by the same network of venous capillaries. These facts have been known for many years (Ryder, 1882, 1884, 1885; Wenckebach, 1886; Hochstetter, 1888), but

* Prof. R. R. Humphrey of the Anatomy Department of the University of Buffalo kindly supplied me with several dozen partial albino *Amblystoma* eggs on which I was able to confirm the venous supply of the yolk sac capillary net by direct observation of the flow of blood in the living and by vascular injections.

Legend to Fig. 1

Fig. 1. The general circulatory sequences of developing and adult Vertebrata. (1) Condition at first establishment of the circulatory cycle in all vertebrate embryos. (2) Circulatory sequences in early vertebrate embryos at about the time of the first appearance of somites. Note the similarity between Selachii and Amniota at this stage, and the marked difference between these and the Osteichthyes and Amphibia where the body and gut are interposed between the gills or visceral arch region and the yolk sac. (3) The late embryonic or foetal condition, except in Amniota where the period represented is about the limb-bud stage at which the primary organ systems are just established. (4) The late embryonic and foetal stage of Amniota, the period of the functional metanephros and allantois. Top row and right column: adult circulatory sequences of the chief representatives of the various major groups of vertebrates. — arteries, ━ veins, ▥ veins which are probably not present in all species of the group, ‖‖‖ veins of minor size, ± structures which may, or may not, be present, such as placentation in the case of the yolk sac and allantois in Amniota, ( ) structures apparently non-functional at the stage shown, x pulmonary vein, present in Cladista, Dipneusti and *Amia* only, y absent in Cladista, Dipneusti and *Amia*. No attempt has been made to indicate the partial separation of the pulmonary circulation in the heart and ventral aorta in Cladista and Dipneusti. z veins absent in Marsupialia and Eutheria.

were largely forgotten until recently called to mind by Turner's studies (Turner, 1940).

In the later embryonic and the foetal stages the venous supply to the yolk sac and pericardium of fishes and amphibians is maintained as long as these membranes persist in their characteristic embryonic condition (Fig. 1, stage 3).* This of course does not mean that all body drainage must pass through the yolk sac. The anterior cardinals and the subintestinal frequently drain wholly, or in part directly, into the sinus venosus, and the posterior cardinals may also drain in part directly to the heart.

The efferent drainage of the yolk sac in all forms is at first directly into the venous end of the heart. (In the bony fishes definite paired vitelline veins may never be clearly differentiated.) However, a little later (Fig. 1, stage 3) the sinusoidal capillary system of the liver is insinuated between the yolk sac and the heart in such a way that practically all of the vitelline blood must pass through the liver sinusoids before reaching the heart. In reptiles and birds, and some mammals, a shunt eventually forms from the umbilical vein straight through the liver to a hepatic vein (the meatus venosus of reptiles and birds, ductus venosus of mammals) (Lillie, 1940). It carries not only a large part of the umbilical (allantoic) blood, but also at least some of the blood from the vitellines which at this time drain both the yolk sac and developing intestines.

Allantois. Only in the later embryonic and the foetal period of amniotes is there an allantoic circulation. It is always supplied by umbilical (allantoic) arteries which are direct branches of the aorta. Its drainage is at first directly into the sinus venosus by way of the umbilical (allantoic) veins, but later is wholly or in part through the liver sinuses. The umbilical veins eventually drain a portion of the bladder and ventral abdominal wall and are often considered homologous with the anterior or lateral abdominal veins of anamniotes. (For further details see discussion under *Liver*.)

Liver. A hepatic portal system is of universal occurrence in vertebrates. It develops concomitantly with the liver in the late embryonic period, for it is formed by the invasion of the liver cords into the proximal portions of the vitelline and umbilical (allantoic) veins in the amniotes, and the vitellines, subintestinals, and anterior or lateral abdominals in the anamniotes. In all, then, there is a period during the later embryonic stages when the venous blood from the intestine and the yolk sac drains through the hepatic sinuses before reaching the heart. In most bony fishes the veins from the cephalic end of the swim-bladder drain into the hepatic portal, and those from the gonads often do also, although these latter may drain in part or entirely into the cardinal system. In the amniotes the allantoic blood is also shunted through the liver by connexions which form between one or both of the umbilical veins and the intrahepatic net originally derived from the vitellines. In birds and reptiles, where the allantois is primarily respiratory and excretory in function, this connexion is ordinarily merely a shunt through the meatus venosus directly to the hepatics and inferior vena cava. In mammals, where the allantois has in addition a very important nutritive function, its venous blood during the early embryonic period drains entirely through the liver sinuses. In many of this class, as for example the horse and pig, this condition persists to term. However, in others, such as man, a ductus venosus develops, serving, like the meatus venosus of birds and reptiles, to shunt much of the umbilical blood directly to the hepatic veins and inferior vena cava.

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* I have not checked the blood supply of the parietal pericardium in the adults of these forms, but it is probable that it is arterial.

A nutritive hepatic arterial supply probably exists in all vertebrate animals.

In most vertebrates anterior or lateral abdominal veins drain cephalad along the ventral or lateral portions of the abdominal wall (Gelderen, 1931). Typically these receive some blood from the caudal region, posterior extremities, and urinary bladder, as well as from the abdominal wall itself. In bony fishes (Allen, 1905; Spencer, 1893) and Selachii (Parker, 1886; O'Donoghue & Abbott, 1928; Marples, 1936) this system drains past the liver to the heart. In Amphibia (Bethge, 1898; Reese, 1906) and Reptilia (Stromsten, 1905; Reese, 1915; O'Donoghue, 1920; Ray, 1936) it drains into the liver sinuses by way of the proximal end of the hepatic portal. In birds (Hochstetter, 1888; Spanner, 1939) and monotremes (Beddard, 1884) there is a similar vein, called respectively the epigastric and anterior abdominal, draining only the bladder and a small part of the abdominal wall into the hepatic portal. In marsupials and eutherian mammals there are only very small anastomosing channels in the tissue of the falciform ligament of the liver (para-umbilical or Sappey's veins) which connect the 'superior epigastric-internal mammary' system with the hepatic portal. In these latter the main drainage of the anterior abdominal wall is through the epigastric and internal mammary vessels to the venae cavae, thus by-passing the liver. The para-umbilical veins of mammals are probably not remnants of the umbilicals, but are merely local veins of the ventral mesentery of which the falciform ligament of the liver is a vestige. The ligamentum teres of the liver is the remnant of the left umbilical vein.

Kidneys. There is still considerable confusion about the comparative anatomy of the renal portal circulation. If we mean by a renal portal system the breaking up of large afferent renal veins into a capillary or sinusoidal net in the substance of the kidney, the net in turn being drained by efferent renal veins, then there is apparently sound anatomical evidence that a true renal portal circulation exists in adult Selachii, Osteichthyes, Amphibia, Reptilia, and Aves, as well as in the embryonic and foetal stages of these and of Mammalia (Jourdain, 1859; Hochstetter, 1893). However, Spanner (1929, 1939) has shown that large venous anastomoses occur between the afferent and efferent renal veins of certain urodeles, reptiles, and birds, so that a large portion of the so-called renal portal blood may be shunted past the nephric capillary bed. Ryke (1926) and Möllendorf (1930) showed that the painted turtle (*Chrysemys marginata*) does not have these anastomoses, but that the snapping turtle (*Chelydra serpentina*) does. The presence and the variability of these shunts are things which one might expect, as they are entirely in line with the general pattern of the vascular system, and in fact have an almost exact counterpart in the ductus venosus and meatus venosus of the hepatic circulation of foetal mammals and birds. It should also be pointed out that in most vertebrates a significant portion of the venous blood from the posterior part of the body by-passes the kidneys in one way or another (via lateral or anterior abdominals, posterior cardinals, vertebrals, etc.); so from the physiological viewpoint there is no obvious reason why a shunt should not occur in the immediate vicinity or substance of the kidneys. In this connexion it is, however, very significant that Spanner (1939) has shown the presence of specialized valve and sphincter mechanisms in these anastomosing renal vessels in birds. This, of course, very strongly suggests a physiological regulation of the amount of blood passing through the portal capillaries.

In a very challenging paper Woodland (1922) feels that he has demonstrated that the renal portal sinusoidal plexus of the frog (*Rana tigrinum*) has no anatomical or physiological relation to the renal tubules. He therefore maintains that there is no significance in the system, that it is purely an accident of development, and that it is physiologically useless. Such revolutionary findings and conclusions need confirmation by others before being accepted. It is hard to believe that a system of capillaries or sinusoids or even fine veins, as intimately related to an organ as is that of the renal portal to the kidney, has no functional relation to that organ. It is of course known that the excretory function of the kidney is not its only one.

Another peculiar feature of the renal portal system occurs in certain fishes (Bridge, 1904; Allen, 1905), reptiles and birds (Spanner, 1929, 1939) in the form of rather large anastomoses of the renal portal with the hepatic portal system by way of veins connecting the caudal end of the posterior mesenteric vein with each renal portal vein. In some species Spanner thinks blood may flow in either direction in these vessels. Ordinarily the flow seems to be from renal portal to mesenteric.

Gills. There are no essential anatomical or developmental differences among vertebrate embryos in the position of the vessels of the visceral arches in the circulatory sequence. Even in Amniota, where the gill structures probably have no respiratory function, practically all the blood from the heart first passes through the aortic arch system. The main anatomical difference is that in the Amniota the capillary plexus in the visceral arches is very rudimentary and temporary, probably homologous with the initial condition in branchiate embryos before the functional gills have formed, whereas in all the others it is well developed during at least part of the embryonic period and in the adults of most aquatic species.

Lungs and swim-bladder. The lungs of tetrapods are always supplied by pulmonary arteries carrying venous blood, and by a very much smaller nutrient bronchial arterial supply carrying oxygenated blood. Both the pulmonary and bronchial systems arise at an early period in development. Furthermore, the lungs of both adults and embryos are drained by veins leading directly to the heart, never by efferent arteries as are the gills. In other words, the functional and nutritive tetrapod pulmonary circulation is always an independent side circuit, whereas that of the gills is always interposed on the main line from the heart to the body (and extraembryonic membranes, if they are present). Where both gills and lungs are present in tetrapods the pulmonary arteries branch off before the gills are reached but the nutritive bronchial arteries come off the dorsal aorta or one of its branches.

Whatever the respiratory significance of the swim-bladder, it is supplied by blood which has already passed through the gills. In the typical teleost the swim-bladder has an arterial supply to its cephalic (oxygen secreting) end from the coeliac branch of the dorsal aorta, and to its caudal (oxygen absorbing) end by direct branches of the dorsal aorta. The venous drainage of the cephalic end is into the hepatic portal, that of the caudal end into the posterior cardinals. The only known exception to this situation among the Actinopterygii is *Amia*, which has the entire arterial supply to its lung-like swim-bladder from a pair of pulmonary arteries arising from the last pair of branchial efferent arteries (4th branchial, i.e. 6th embryonic or 'visceral' aortic arch), and its venous drainage by right and left veins which join and enter the left common cardinal near the heart. *Gymnarchus* has a similar arterial supply in the embryo, but is like typical teleosts when adult. In one of the dipnoans, *Protopterus*, the two pulmonary arteries arise from the corresponding roots of the dorsal aorta, and so receive blood from all the efferent branchials. In the other dipnoans (*Neoceratodus* and *Lepidosiren*) and in the Cladista (*Polypterus* and *Calamoichthys*) the pulmonary arteries arise as branches of the 5th and 6th aortic arches (*Lepidosiren* and *Calamoichthys*), or of the 6th alone (*Neoceratodus* and *Polypterus*), so that the lungs receive only blood which has passed through the most posterior gill-bearing arches. Only in *Protopterus* are the 3rd and 4th arches gill-less, and on this account it looks at first as though in this genus the lungs might receive mixed blood; but, if the separation of pulmonary vein blood in the heart and ventral aorta is done as efficiently as the elaborate septal mechanisms indicate, then the 3rd and 4th arches carry oxygenated blood anyway, so that only when the fish is in stagnant water would there be mixed blood in the pulmonary arteries, and then it would come from the 5th and 6th arches. Robertson (1913) says, 'In *Lepidosiren* all the aortic arches can be traced throughout as definite uninterrupted vessels.' If this is true, there should be mixed blood in the pulmonary arteries of this genus.

In pulmonary venous drainage the Cladista, Dipneusti, and *Amia* are fundamentally alike. In *Polypterus* the pulmonary veins drain into the hepatics, and in *Calamoichthys* into the posterior vena cava very near the heart. In the dipnoans they always drain into the left atrium, and in *Amia* to the left common cardinal. In all other Actinopterygii, so far as known, the veins from the cephalic oxygen-secreting portion of the swim-bladder enter the hepatic portal, and those from the caudal oxygen-absorbing part drain into the posterior cardinals, often in part at least by way of the renal portal system.

References for the pulmonary blood supply of the various groups are the following: general—Boas (1880), Bridge (1904), Kerr (1919), Ballantyne (1927), Ihle, Kampen, Nierstrasz & Versluys (1927), Goodrich (1930), and Grodziński (1938); *Polypterus*—Budgett (1901) and Rauther (1922); *Calamoichthys*—Purser (1926, 1928); *Neoceratodus*—Spencer (1893), Kellicott (1905); *Lepidosiren*—

Robertson (1913); *Protopterus*—Parker (1892); *Amia*—Kingsley (1885); Actinopterygii—M'Kenzie (1883-4), and Allen (1905).

Other organs. Just as there are additional details and exceptions to the conditions of circulation already outlined for various organs, so there are many additional organs and regions which have interesting and probably significant positions in the circulatory cycle. Among these are the drainage of the stomach, spleen, and pancreas into the hepatic portal; the afferent and efferent arteries of the kidney glomeruli; the rich cutaneous supply of amphibians with its respiratory function; the suprarenal portal of many lower forms; the supply of the adrenal medulla of mammals largely by blood which has passed through the capillaries of the cortex; the pituitary portal (Green, 1947); and doubtless many others. For the sake of conservation of time and space these more special and probably less fundamental examples will not be discussed.

III. DISCUSSION

Yolk sac. It is well known that the first blood vessels arise in close association with the yolk sac in all vertebrates. It is generally believed that both the vessels and the corpuscles develop from the visceral mesoderm of this region, although there is some evidence that the endothelium only is of mesodermal origin and that the first corpuscles are endodermal (Ryder, 1882, 1884; Gladstone & Hamilton, 1941). It is obvious then that if the vitelline net develops from the splanchnopleure its vessels should be closely associated with those of the midgut. This is of course the situation in the case of the vitelline vessels of the elasmobranchs and amniotes, but in the bony fishes and amphibians the situation is very different. In these the vitelline net is associated with the caudal and cardinal veins, both of which are essentially vessels of the somatopleure, and to a lesser extent with the subintestinal. This condition probably can be partly explained by the fact that in bony fishes and amphibia the differentiation of the vitelline net precedes the extension of the coelom into the region. The cardinal veins collecting blood from the short intersegmental branches of the dorsal aorta are nearer the yolk sac than the arteries themselves, and apparently develop before visceral branches of the aorta. Thus they are in a position to lose blood to the yolk sac net, particularly since there is as yet no coelomic space in the region to make this connexion difficult. The caudal vein is in an especially favourable position, in the absence of a coelom near the proctodeum, to drain directly to the yolk sac net and later to the intestinal net when it develops. An anastomosis between the caudal, the subintestinal, and the posterior cardinals in these animals is common. In certain pelagic fishes with small relatively yolkless eggs the caudal vein is extended without capillary interruption along the midventral side of the yolk sac to the heart (Kryžanovsky, 1934). This condition may be a precocious formation of the ventral abdominal vein or its homologues, a system characteristic of the adult fish and amphibian. The best conception of the yolk sac in these forms, then, seems to be that it develops from the first as a relatively unmodified portion of the body wall anticipating the relatively early stage of development when the yolk is used up and the yolk sac does become true body wall.

In *Gymnarchus* the yolk sac is at first large and pendent, but it is eventually absorbed into the belly wall just as in other teleosts (Kerr, 1919). In the selachians and amniotes there is usually either much more yolk or, as in mammals, the function of the yolk sac is much more highly specialized. Furthermore, in these its period of existence relative to the degree of anatomical maturation of the embryo is much longer. All of this seems to require that essentially the whole ventral body wall should develop during the existence of a functional yolk sac, and so instead of developing from the definitive yolk sac the ventral wall is of necessity formed from other elements; the sac itself is finally either resorbed or 'amputated' when its specific functions terminate.

It appears then that the yolk sac of bony fishes and amphibians is from the first truly a part of the ventral body wall, and remains so, whereas that of selachians and amniotes is a much more widely modified portion of the embryonic body wall and gut wall and is largely shed, never contributing more than a very minor amount of tissue to these structures of the adult. In the sharks it remains a combined somatopleuric and splanchnopleuric structure, but in amniotes it usually passes through unilaminar (ectodermal), bilaminar (ectodermal-endodermal), and trilaminar (ectodermal-mesodermal-endodermal) stages relatively early and in quick succession; and then, as the exocoelom develops, the splanchnopleuric portion (definitive yolk sac) is rapidly and widely separated from the somatopleuric portion, the latter becoming a major part of the true chorion.

Structurally, developmentally and probably physiologically the yolk sacs of bony fishes and amphibians are fundamentally alike and at the same time very different in all these respects from those of Selachii and Amniota. Between the Selachii and the Sauropsida (reptiles and birds) there is certainly a wide anatomical gap in yolk sac morphology, because of the absence of the amnion and chorion in the former. However, the nature of the circulation, the fate of the splanchnopleuric portion, and the physiology in the two groups are essentially the same. Mammalia, of course, have yolk sacs in general very similar in structure and blood supply to those of Sauropsida, but because of the minute amount of yolk in the eggs of all except the Monotremata the ontogeny and physiology are very different.

We have, then, besides the ontogenetic and physiological problems relative to the vitelline structures of vertebrates, the problem of interpreting the meaning of all these from the standpoint of the evolutionary and phylogenetic relations of and within the whole subphylum.

It is obvious, from the blood supply and other features, that there is a much closer affinity between the yolk sacs of the Osteichthyes and the Amphibia than there is between either of these and the yolk sac of Selachii. In fact, the yolk sac of Selachii is more like that of the Sauropsida than it is like that of the lower groups. Of course there is much in common in the vitelline structures of all the Amniota, in spite of the great variety of methods of yolk sac formation and of definitive form among Mammalia.

The easiest and simplest interpretation of all this is to assume that these degrees of difference and similarity indicate degrees of phylogenetic relationship and lines of evolution. While I am inclined to think that such an interpretation does come rather near the truth, I know that the validity of it could be seriously questioned on many points. Perhaps adaptation to a similar environment by primitive amphibians and fishes led to the production in each of eggs with a medium amount of yolk (medialecithal), and this, along with similar environment of the eggs during development, resulted in ontogenetic adaptations which are parallel or convergent in the two groups.

Physiologically, the yolk sac appears to be primarily a nutritive organ, at least in the majority of vertebrates, yet many of its modifications in mammals are of doubtful function; in some cases it is a haemopoietic organ, in others it probably serves some unknown additional purpose (Noer & Mossman, 1947). It is, therefore, interesting to take note of the almost kaleidoscopic progression of structural changes which occur in the development of the yolk sac in any given species, as well as the wide range of variability in the developmental and definitive conditions of the organ between and within the classes of vertebrates.

From the physiological standpoint one of the most fundamental differences of this sort is the venous blood supply of the yolk sac of fishes and amphibians as contrasted with its arterial blood supply in all the other classes. No one knows the significance of this difference. In considering an attack on the problem one should keep at least the following considerations in mind: (1) The yolk sac of both bony fishes and amphibians, as well as that of selachians and sauropsidans, contains a considerable amount of yolk, and is therefore a nutritive organ. (2) The vessels of the yolk sac are for a time at least in a favourable position to serve as an area for aeration of the blood. (3) Haemopoiesis and blood-vessel formation occurs in all vertebrates in the area of the yolk sac, in fact it is usually the first area in which this occurs. (4) Only rarely has the first formation of blood cells in vertebrates been reported elsewhere than in the immediate vicinity of yolk sac endoderm (Hertig, 1935). The secondary and often definitive areas of erythropoiesis (liver and spleen) are in the main derived from splanchnic mesoderm closely associated with endoderm. Only in those vertebrates where bone marrow has taken over this function is the close association of endoderm not apparent. (5) Venous blood entering the yolk sac capillaries of a bony fish or amphibian must contain various metabolic products and possibly metabolic enzymes derived from the body tissues through which it has just passed, in addition to its relatively high content of carbon dioxide and ordinary nitrogenous wastes. The arterial blood entering the yolk sac plexus of other vertebrates must be appreciably different.

All we can do, with the information at present available, is to wonder what these differences really are, speculate on their extent, and on what effect they have on the processes of yolk absorption and transport mechanisms, erythrogenesis, and so forth, which are occurring on the various vitelline sacs. One fairly obvious possibility, but purely a guess, is that the fish and amphibian yolk sac functions somewhat like the liver in detoxifying or excreting poisonous substances carried in its portal blood supply. It may be that selachian and other embryos deal with this problem in some other way. Of course it is possible that whether a particular vertebrate group has an arterial or a venous vitelline blood supply is purely an accident of developmental phylogeny, and that it has no functional import; but it seems much more likely that there are fundamental physiological problems involved, and that it is an indication of a close phylogenetic relation between Osteichthyes and Amphibia and of a wide gap between these two and other Vertebrata.

Allantois. As a nutritive and respiratory organ the allantois is a much more simple problem than the vitelline sac. Its blood supply is always arterial and its drainage is always, in part at least, through the liver sinuses. In all allantois-possessing vertebrates some sort of by-pass of the liver sinuses is provided for allantoic venous blood, with the known exception of only a few mammals, including the horse and the pig. This shunting past the liver of the allantoic blood of reptiles and birds, where its function is primarily respiratory, is readily understandable on a functional basis. In mammals, where it has an added purpose of passing nutritive substances to the embryo from the placenta, it is reasonable that some of it should pass through the liver sinuses for the purpose of temporary storage of carbohydrates. The amino-acids carried by the umbilical vein are presumably detoxified in the maternal liver before ever reaching the placenta, so this could hardly be a reason for the hepatic portal circulation of placental blood. Why some mammals, such as the horse and pig, do not have a ductus venosus is not clear. It may be in some way correlated with their epithelio-chorial placentation.

The phylogenetic development of the allantois is a puzzling matter. The usual interpretation is that it arose in the embryonic stages from the ventral bladder of some primitive ancestral amniote. It undoubtedly evolved as an extra-embryonic structure later than the chorion and amnion. Its projection through the incomplete ventral abdominal wall of the early amniote embryo is not difficult to understand. Its veins, the umbilicals, are believed to be homologous with the ventral or lateral abdominal veins of many anamniotes, which typically receive twigs from the bladder and drain into the hepatic sinuses along with the portal. As a matter of fact, the umbilical veins of amniote embryos do serve as veins of the ventral abdominal wall, lying in it alongside the umbilical region and receiving small tributaries from it. (For further discussion of the anterior abdominal veins see under *Liver*.)

Liver. The position of the liver sinuses in the route of the venous return from the absorptive part of the alimentary tract is common to all vertebrates. The chief variable in its relation to the circulatory sequence is in the amount of blood, other than alimentary tract blood, which also flows through it. Attention has been called previously to the fact that in Osteichthyes, Selachii, Marsupialia, and Eutheria, very little other blood enters the hepatic sinuses, that a little body wall and urinary bladder blood enters them in Aves and Monotremata, and that a rather large amount of abdominal wall, posterior extremity, and caudal blood flows through the liver in the Anura, Urodela, and Reptilia.

Stage 3 of Fig. 1 shows blood from the posterior part of the body draining through the liver in larval or foetal stages of all vertebrates, but in more limited amounts in amniotes. For some forms the record is not entirely clear, and one must still question whether somatic drainage to the hepatic portal does or does not exist during development in all cases in Osteichthyes and Selachii where there is practically no such drainage in the adult. Instead of an anterior abdominal type of hepatic supply, the subintestinal is often directly continuous with the caudal, and in that way the hepatic portal system has as one of its large tributaries the veins from the caudal region. Probably the inconstant anastomosis between the posterior mesenteric and the renal portal in adult bony fishes, reptiles, and birds is a remnant of this early connexion. From its inconstancy in the adult it seems that there is probably no definite physiological reason for this particular connexion of somatic veins to the hepatic portal system.

The pattern of the anterior abdominal veins, either as a single median structure, or as a pair of vessels, in adult Amphibia, Osteichthyes, Selachii, and Reptilia; and of its probable homologue, the umbilicals, in Aves and Mammalia, are so relatively similar that one cannot but wonder why the relation to the liver is so variable. It seems most reasonable to assume that the anterior abdominals are in their most primitive condition in Osteichthyes and Selachii, where they flow directly to the heart. In Amphibia the system seems to have reached its height of development, so far as its relation to the liver is concerned. In Amniota it appears to become progressively more modified and reduced in significance in the adult; possibly owing to the mechanics of developmental rearrangement to meet the needs of the allantoic (umbilical) circulation. The most problematic thing about the anterior abdominal system, when all is said, is the question of why it ever became part of the hepatic portal system in the first place. There is no hint of a functional advantage. About the only explanation is that it is an accident of development due to the growth of the liver in the region of the septum transversum which provides thereby a large area of low-pressure sinuses so near to the cephalic ends of the anterior abdominals that they capture the drainage of the latter by offering a lower resistance flow-way to the heart. This appears to be just what happens in mammals when the development of the liver cuts the direct connexions of the vitellines and umbilicals with the sinus venosus.

The functional significance of the liver sinuses in the path of the drainage of the venous blood from the absorptive portion of the intestine is too well known to need comment. However, no appreciable amount of glucose or non-detoxified amino-acid is absorbed from the stomach or large intestine in any vertebrate, yet these regions almost always drain to the hepatic portal. Is this merely topographically convenient, or is some obscure function being served? Could it be that there are hormones produced in the gastric wall, or vitamins by the flora of the colon with which the liver has to deal? It is known that the liver is stimulated to secrete bile by the presence of pancreatic juice in the duodenum. That the liver may have important functions in dealing with certain enzymes and hormones is suggested by recent work (Jungck, Heller & Nelson, 1947) showing that ovarian and testis grafts, placed where their blood drains through the hepatic portal, have no effect on the pituitary, presumably because their secretions are destroyed or inactivated in the liver. This is not at all surprising considering the many known functions of the liver in such complex processes as urea synthesis, bile salt production, the breakdown of haemoglobin from the spleen, etc. One is therefore almost forced to conclude that there are probably many still undiscovered functional reasons for the uniform position of the liver in the circulatory sequence in the vertebrates.

Kidneys. The fact of the existence of a renal portal system, in either the developmental stages or the adults of all vertebrates, strongly suggests that the insertion of the kidneys into the circulatory organ sequence between part of the venous return from the rear of the body and the heart is of fundamental physiological import. The rather common existence of shunts between the afferent renal portal veins and the afferent renal veins in amphibians, reptiles and birds, and the entire absence of a renal portal in adult mammals suggest a tendency to do away with whatever the physiological mechanism is in certain groups. Obviously it is utilized only during embryonic development in mammals, and then completely eliminated. For years an excretory function was assumed for it, but Woodland's findings throw doubt on this (Woodland, 1922), and open up the whole problem again. The present interest in non-excretory functions of the kidney, especially the part it plays in hypertension, should stimulate research on forms having a dual renal blood supply.

From a functional viewpoint it is interesting that in bony fishes gonadal veins and some veins from the swim-bladder often drain into the renal portal sinus system; also in the region of the head certain veins draining part of the brain and spinal cord may pass through a 'very fine venous plexus' in the head kidney (Allen, 1905). However, it should be remembered that the head kidney of Osteichthyes metamorphoses during larval life into a peculiar lymphoid organ (Leydig's organ) which persists as such in the adult where it shows no evidence of an excretory function. Of course the more caudal portion of the spinal cord commonly drains into the caudal vein and thence to the renal portal in all forms possessing this system.

Another peculiarity which may have some significance is the somewhat inconstant but often rather large anastomosis between the renal portals and posterior mesenteric vein, described in Osteichthyes (Allen, 1905), Reptilia (Spanner, 1929), and Aves (Spanner, 1939). The flow is probably from renal portal to the hepatic portal, according to Spanner. This has already been discussed briefly under 'kidney' in the anatomical section, and does not warrant further space, as so little is known about it.

From the ontogenetic standpoint it may be significant that in those vertebrates in which a marked caudal migration of the gonads (especially the testes) takes place, the renal portal system disappears. Apparently this is correlated with the intimate connexion of the mesonephros with the gonad and the consequent migration of this portion of the nephros together with the gonad, as well as with the concomitant degeneration of the mesonephros as an excretory organ. The pampiniform plexus is in part a remnant of the renal portal venous plexus, although it may represent the larger anastomosing veins rather than the sinus plexus itself. In the birds and reptiles, where gonad migration is slight, the distinction between mesonephros and metanephros is never as evident as in mammals, and it is in these two groups that a partial renal portal system persists in what appears to be a metanephric type of kidney.

It is of interest that the renal portal system is as much of a common character of all vertebrates as the branchial arch system. Whatever its significance may be physiologically, it is certainly an ancient and universal characteristic of Vertebrata.

Gills. The position of the gills in the circulatory sequence is an obvious one in that they receive blood directly from the heart and aerate it before it goes to the rest of the body. In amniotes the rudimentary condition of the gills in the embryo and early moulding of the vascular net of each branchial arch into a simple aortic arterial channel makes it quite clear that they are practically functionless so far as respiration is concerned. The branchial apparatus of this group is perhaps the most clear-cut example of vestigial organs which have none of their ancestral physiological function, but have been retained because they are necessary building blocks for other structures. This apparatus is also one of the most characteristic features of the Vertebrata as a whole, indicating (along with the vertebrae themselves, the tubular dorsal nervous system, etc.) the common phylogenetic relationship within the group.

Lungs and swim-bladder. As was pointed out before, the pulmonary circulation of the typical tetrapod lung is always in the form of a separate complete arterial-capillary-venous cycle; never is it, like the gill circulation, merely a capillary bed inserted into the arterial pathway to the body capillaries. The changes in the circulatory system, particularly in the heart and great vessels, that go with the introduction of the pulmonary circuit are one of the most remarkable things in the field of comparative morphogenesis and evolution. Also they are of great value as criteria of phylogenetic relationships between the subgroups of vertebrates, because the pulmonary system evidently developed well after the more generalized vertebrate characters were established.

The swim-bladder of fishes and the lungs of the lung-fishes are certainly much more comparable to one another than they are to the typical tetrapod lungs as found, for instance, in amphibians. This fact is if anything more obvious in their vascular sequence patterns than it is in the structure of the organs themselves. The swim-bladder or lung-fish lung is never on a separate circuit as tetrapod lungs always are. That the separate circuit type of lung could have evolved from the swim-bladder type seems somewhat doubtful, but this is not the place to enter into this problem (Ballantyne, 1927).

A peculiarity of the swim-bladder in the circulatory sequence, at least in certain fishes, is that its rear portion often drains into the renal portal veins. Of course this may be purely fortuitous.

Although the function of the lungs is too well known to warrant any discussion here, that of the swim-bladder and lung-fish lung is not. As a static organ the particular position of the swim-bladder in the circulatory sequence is unimportant.

However, the swim-bladder of many fishes is now known to serve the purpose of a 'reserve tank' for oxygen storage, and to some extent as a 'receptacle' for carbon dioxide during periods when the individual is unable to carry on sufficient exchange through the gills. It should be, and therefore is, supplied with arterial blood which has just passed through the gills. The fact that the lung-fish lungs occupy exactly this same position indicates that they also have a storage function, although it is to a certain extent coupled with a real lung-like respiratory purpose.

In other words, the lung-fish lung is enabled, through the gulping of air into it and the belching of gases out of it, to serve as an area of direct interchange between the air and the blood, but at the same time its supply by blood, which has first passed through the gills, also enables it to function as a storage or reserve tank by taking oxygen from the blood. This is a mechanism characteristic of the swim-bladder but essentially absent from the tetrapod lungs as seen in Amphibia and higher forms. They may well store inspired air to a certain extent, but they do not store oxygen acquired by the blood through some other respiratory organ and secreted into the lung from the blood.

IV. GENERAL CRITIQUE

A critical retrospect of this article may be worth while at this point. In the first place, details of anatomical fact have been omitted whenever possible in order to emphasize the most important points. Secondly, appreciation of many accessory considerations has been taken for granted; as, for instance, the presence of anastomosing connexions in the vascular systems of all vertebrates, which means that a completely independent vascular circuit to an organ is almost never an actuality; or perhaps, even more important, the presence of the lymphatic or 'third' circulation. The significance of the latter is often great, as, for example, in the case of the abdomen where this system allows absorbed fat completely to by-pass the liver on its way to the heart. Thirdly, I have committed myself to an opinion on very few 'theoretical' points, because the evidence at hand usually seems to be insufficient for sound judgement. Fourthly, consideration of certain groups such as cyclostomes and protochordates has been omitted partly because of their questionable significance, and partly because of lack of the necessary information in certain cases. Fifthly, only one or two references are given for most of the points discussed, because without rigid selection the bibliography would have been enormous.

The justifications for writing this article seemed to me to be numerous. In the first place there was an idea involved which, while probably not new, at least did not seem to have been presented in recent years; an idea which it seemed might stimulate productive thought and research, using as its tools comparative anatomy. This idea had come from facts acquired during long study of the comparative morphogenesis of the foetal membranes of vertebrates, and being a 'side-line' which could not be followed far enough without straying too widely from my major interests, it seemed best to dispose of it as a separate issue. I have also hoped that some of the discussion, and especially the diagrams and references, would prove to be an aid and a stimulus in the teaching of comparative anatomy.

V. SUMMARY

1. The following significant facts have been pointed out:

(*a*) The yolk sac of Osteichthyes (bony fishes) and Amphibia is supplied by blood from body veins, while that of Selachii (cartilaginous fishes) and Amniota receives blood by way of direct branches from the dorsal aorta.

(*b*) The allantois is always supplied directly from the aorta by the umbilical arteries. Its venous drainage is at first by way of the umbilical veins directly to the heart, but later part or all of it passes through the liver sinuses.

(*c*) A hepatic portal system exists in all Vertebrata, but in developing Amniota a by-pass around the liver sinuses is provided in Aves and Reptilia by the meatus venosus, and is often provided in Mammalia also by the ductus venosus. These structures allow the allantoic vein blood and some of the vitelline and intestinal vein blood to reach the heart directly. Anterior abdominal wall and urinary bladder blood and some from the caudal region and posterior extremity drain into the hepatic portal and liver sinuses in Amphibia and Reptilia, and to a slight extent in birds and monotremes through the anterior abdominal vein or its homologue.

(*d*) A renal portal circulation is present in all Vertebrata during embryonic life. It is present in the adults of all except Mammalia. It is apparently modified by the presence of venous anastomoses between afferent and efferent renal veins in many of these. Its anatomical and physiological relation to the renal tubules has been denied by at least one recent investigator.

(*e*) The gills always receive blood directly from the heart by way of the ventral aorta and are drained by efferent arteries leading directly to the roots of the dorsal aorta. Head arteries arise from some of the efferent branchials, and in certain fishes pulmonary arteries also arise from them. Typical tetrapod lungs receive their functional supply by arteries directly from the heart or ventral aorta, and drain by veins directly into the heart. On the other hand, the lungs of lung-fishes and the swim-bladder of other fishes receive blood either from the dorsal aorta or from branches of the efferent branchial arteries and drain into both the systemic venous system (sometimes in part via the renal portal) and the hepatic portal in most fishes, and more or less directly to the heart in *Amia*, Cladista and Dipneusti.

2. The following possibilities and problems have been discussed:

(*a*) The blood supply of the yolk sac of Osteichthyes and Amphibia is fundamentally different from that of Selachii and other vertebrates, indicating a difference in the physiology of the yolk sac and in its evolutionary development in these two groups. In this respect, then, selachian ontogeny is much closer to that of the amniotes than to that of other anamniotes.

(*b*) The logic in the by-passing of the liver by allantoic blood in those forms where the allantois is primarily respiratory is pointed out, as well as the possible carbohydrate regulating function of the liver in those foetuses (mammals) where the allantois has a nutritive function. Explanation for the complete absence of a ductus venosus in some mammals is lacking.

(*c*) A hepatic portal system is a characteristic of all vertebrates, so far as the conveying of blood to the liver from the absorptive part of the gut is concerned. It is pointed out, however, that in certain groups large amounts of blood from the body wall and caudal half of the body also pass through the liver. The best explanation for this may be that it is an accident of development, for there does not seem to be any obvious function served by this condition. The importance of the drainage of non-absorptive portions of the alimentary tract and other organs through the liver for specific physiological purposes is considered.

(*d*) Despite the universal occurrence of a renal portal system in either the adults or developmental stages of all vertebrates, its physiologic importance is still an unsettled question. Phylogenetically it is certainly one of the fundamental characteristics of vertebrates.

(*e*) The reason for the position of the gills in the arterial circuit from the heart to the rest of the body is obvious. The retention of the vestigial branchial apparatus, including the vessels, in higher vertebrates is one of the best examples of persistence of ancestral structures, not for their primitive physiological function during a transitory period, but because they are the building blocks from which greatly modified specialized parts of the higher forms are constructed.

(*f*) The position of tetrapod lungs in the circulatory sequence, receiving as they do non-aerated blood, is contrasted to that of the swim-bladder and lung-fish type of lung which receive at least partially aerated blood which has already passed through the gills. This is in line with the 'storage tank' function of the latter type of lung and the swim-bladder, as compared to the aerating function of the true lung.

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THE STRUCTURE OF GLYCOGENS

By D. J. BELL, *The Biochemical Laboratory, University of Cambridge*

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I. INTRODUCTION

Glycogen is the name given to the D-glucose-derived polysaccharide found in the cells of animal tissues. While it occurs in considerable amounts in the liver (and homologous organs), and in much smaller quantities in muscle, it is also found in those tissues not ordinarily associated with marked metabolic activities such as the cells of adipose tissues. In all cells glycogen appears to act as a reserve of energy which is made available through the integration of the primary non-oxidative reaction

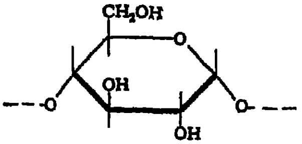
$$\text{Glycogen} \rightarrow 2 \text{ pyruvate} + [4\text{H}],$$

with the secondary oxidative process

$$2 \text{ pyruvate} + [4\text{H}] + 6\text{O}_2 \rightarrow 6\text{CO}_2 + 6\text{H}_2\text{O}.$$

The second process yields far greater amounts of energy than the first and takes place through the mediation of the so-called 'tricarboxylic acid cycle'.

The chemical characterization of a polysaccharide such as glycogen is extremely difficult. Physical measurements indicate that all specimens of glycogen (isolated by the usual methods) are markedly heterogeneous with respect to the masses of the component molecules. Nevertheless, it appears from chemical evidence that all the molecules of all glycogen specimens are built up from the same radical, on what may be termed a common statistical ground-plan.



1:4 Glucosyl radical, α-linked

The usually accepted criteria for characterizing glycogen may be summarized as follows:

(1) High molecular weight.

(2) Iodine solution stains glycogen, in solid form or in aqueous solution. With relatively high concentrations of iodine the colour developed is usually reddish

brown, but careful addition of small amounts of iodine reveals that colours from light yellow-brown to violet may be obtained according to the source of the specimen.

(3) Aqueous solutions have a high dextrorotation, $[\alpha]_D + 190°$ to $+200°$.

(4) The ordinary sugar oxidizing agents, e.g. Fehling's solution, are not appreciably reduced by glycogen.

(5) Weak solutions of mineral acids (e.g. 2·5% HCl) at 90–100° hydrolyse glycogen to D-glucose, in the anticipated yield.

(6) The combined action of the maltogenic ('β') and dextrinogenic ('α') amylases hydrolyse glycogen to give an almost quantitative yield of maltose.

(7) The polysaccharide is free from noticeable amounts of nitrogen and phosphate.

(8) The constituent glucosyl radicals are linked in the 1:4-α manner.

It will be obvious that the above criteria are superficial and can equally well be applied to the amylopectin fraction of native starch, or to α-amylodextrin. The latter is the 'residual dextrin' remaining after the completed action of the maltogenic amylase on amylopectin. Yet these criteria, and too often only a few of them, have been used as the basis for alleged occurrences of 'glycogen'. It may well be that the so-called glycogens said to occur in green plants, fungi and other microorganisms, are, in fact, substances not identical with the glycogens typical of the animal kingdom. Again, the above criteria do not tell us whether the ground-plan of the chemical structure is the same in all specimens.

II. ISOLATION AND PURIFICATION OF GLYCOGEN

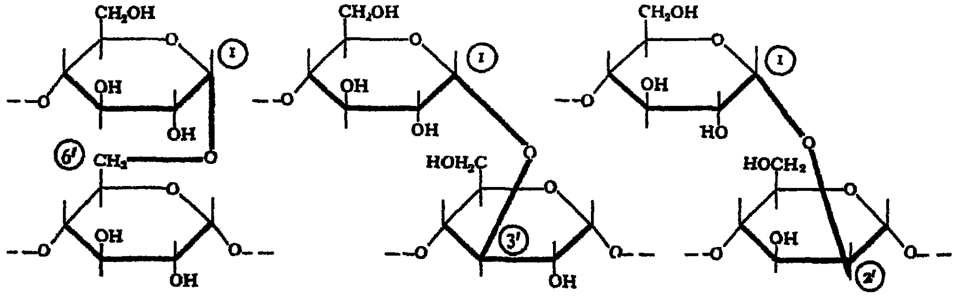
The classical method is that of Pflüger, where the tissue is disintegrated by heating with strong alkali hydroxide solutions. This procedure has frequently been criticized on the grounds that it must lead to degradation of the glycogen molecule. Such measurements as can be made, however (e.g. the molecular weight determinations of Bell, Gutfreund, Cecil & Ogston, 1948), indicate that no detectable diminution in molecular size results from the treatment with strong alkali. The glycogen is isolated by precipitation by ethanol, and is thus obtained in a crude form contaminated by iron, phosphate, silica, etc. Bell & Young (1934) have shown that extensive purification can be readily effected by reprecipitating the crude material from aqueous solution by acetic acid, an agent first used by Claude Bernard. The amount of organic phosphate in preparations purified by this method is negligible (cf. Bell & Kosterlitz, 1935). With some tissues, e.g. liver, it is possible to extract the glycogen by boiling water, or by grinding with ice-cold trichloracetic acid. Here, after deproteinization by picric acid, or by trichloracetic acid, and after reprecipitation by acetic acid, phosphate-free preparations can be obtained.

K. H. Meyer and his collaborators favour extraction of the tissue by chloral hydrate solution to avoid possible degradation of glycogen by alkali. While this procedure appears to be a satisfactory one, I am not satisfied that it has any advantages over the older methods, particularly since, as stated above, I have been unable to demonstrate a degradation effect of alkali.

III. THE STRUCTURE OF GLYCOGEN AS REVEALED BY METHYLATION AND END-GROUP ASSAY

The methylation of glycogen is difficult. Since the molecule is roughly spherical it appears possible that some of the hydroxyl groups in the interior are mutually and strongly hydrogen bonded and thus resist etherification by the methylating reagents. I have not so far succeeded in preparing a sample of methylated glycogen where it is possible to demonstrate that every hydroxyl group has, in fact, been methylated. Despite apparent complete methylation in occasional instances, i.e. when the methoxyl content of the material approaches closely to the value of 45·6 %, subsequent examination of these materials has shown that the high methoxyl content must be due to some analytical error. This point will be more fully discussed later.

From investigation of methylated glycogens we can deduce that the polysaccharide is built up from chains of the afore-mentioned 1:4 α-linked glucosyl radicals. These chains, which may be termed 'unit chains', appear to be composed of relatively small average numbers of radicals; this number can at the present only be deduced on a statistical basis, and is usually known as the 'chain-length'. Moreover, the large molecular weights of glycogens show that the molecules must be built up from some kind of aggregation of several thousands of unit chains. There is no reason to believe that the interchain linkages are other than glucosidic in nature. These interchain glucosidic links must involve the reducing group (position 1) of the radical situated at the beginning of one unit chain, and a hydroxyl group belonging to an intermediary radical of an adjacent unit chain. Three alternatives are possible as shown below, when all the inter-radical links in the chains are of the 1:4 type.



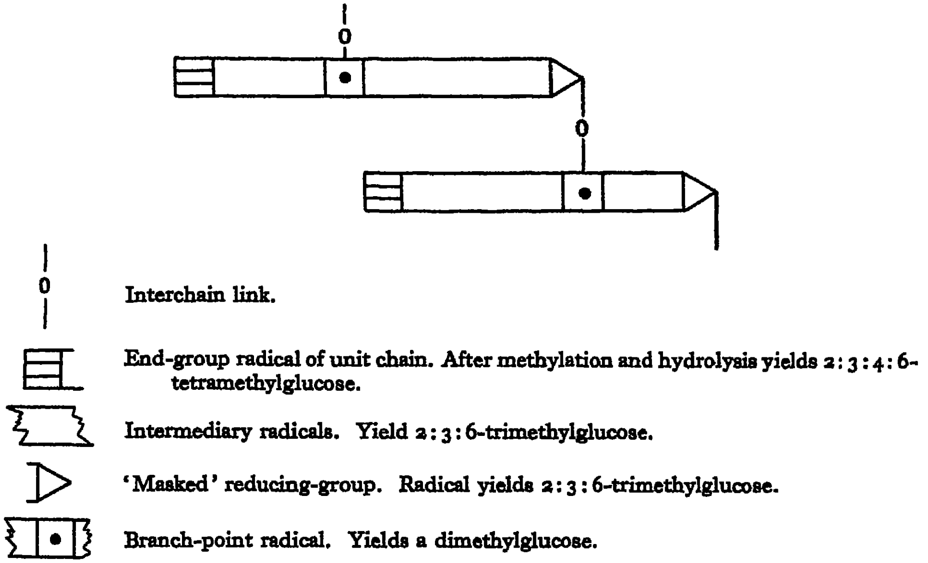
1:6′ type   1:3′ type   1.2′ type

We can represent our ideas of the ground-plan of the structure of glycogen as in Fig. 1.

Inspection of Figs. 1 and 2 will show that methylation of glycogen up to the theoretical maximum, followed by hydrolysis of the methylated material, ought to yield the following products:

(*a*) 1 mol. of tetramethylglucose (in fact, the 2:3:4:6 derivative).
(*b*) x mol. of trimethylglucose (in fact, the 2:3:6 derivative).
(*c*) 1 mol. of dimethylglucose (either of the 2:3, 2:6, or 3:6 derivatives or a mixture).

Thus, theoretically we should obtain for every molecule of tetramethylglucose a single molecule of dimethyl sugar originating in the branch-point radical. In practice, this has not been achieved; the amount of the dimethyl fraction considerably exceeds the proportion of a single molecule relative to the end-group. It is, therefore, not clear at present whether: (1) in a particular sample of glycogen all the branch-point radicals have the same structure (i.e. does the interchain linkage involve position 6, or 3, or 2); or whether they form a random assortment; (2) all glycogens isolated from different animals or tissues have the same type of interchain links.



Interchain link.

End-group radical of unit chain. After methylation and hydrolysis yields 2:3:4:6-tetramethylglucose.

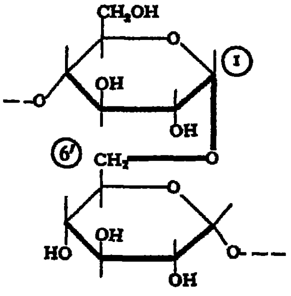
Intermediary radicals. Yield 2:3:6-trimethylglucose.

'Masked' reducing-group. Radical yields 2:3:6-trimethylglucose.

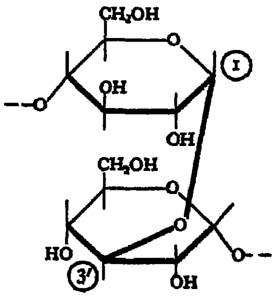
Branch-point radical. Yields a dimethylglucose.

Fig. 1. Diagrammatic representation of the 'branched' structure of a portion of a glycogen molecule.

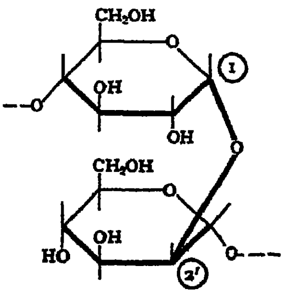
An alternative type of interchain glucosidic link is conceivable. This must of necessity occur only occasionally, and has not so far been detected. This type would involve the *end-group* of a unit chain and three alternatives are possible.



1.6′ type

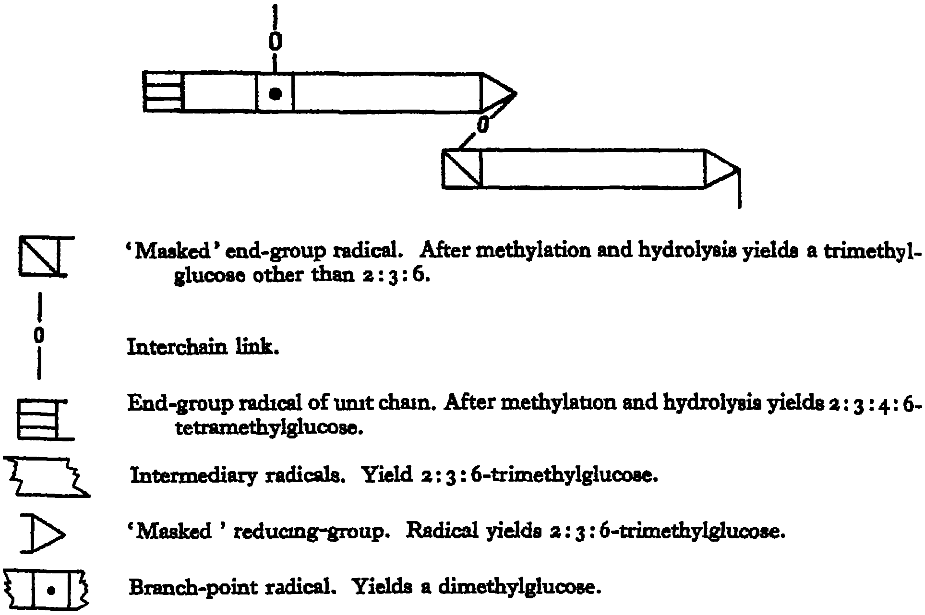


1:3′ type



1:2′ type

With this type of interchain link the branch-point radical would be discovered, after methylation and hydrolysis of the polysaccharide, in the form of a trimethyl sugar molecule having a structure isometric with 2:3:6-trimethylglucose. Thus occasional molecules of 2:3:4-, 2:4:6-, or 3:4:6-trimethylglucoses may occur in the hydrolysis products, but such have so far escaped detection. The elegant chromatographic separation of these compounds devised by Boissonas (1947) should enable this point to be cleared up. Fig. 2 shows the occurrence of such an 'atypical' unit chain as part of a glycogen molecule.



'Masked' end-group radical. After methylation and hydrolysis yields a trimethylglucose other than 2:3:6.

Interchain link.

End-group radical of unit chain. After methylation and hydrolysis yields 2:3:4:6-tetramethylglucose.

Intermediary radicals. Yield 2:3:6-trimethylglucose.

'Masked' reducing-group. Radical yields 2:3:6-trimethylglucose.

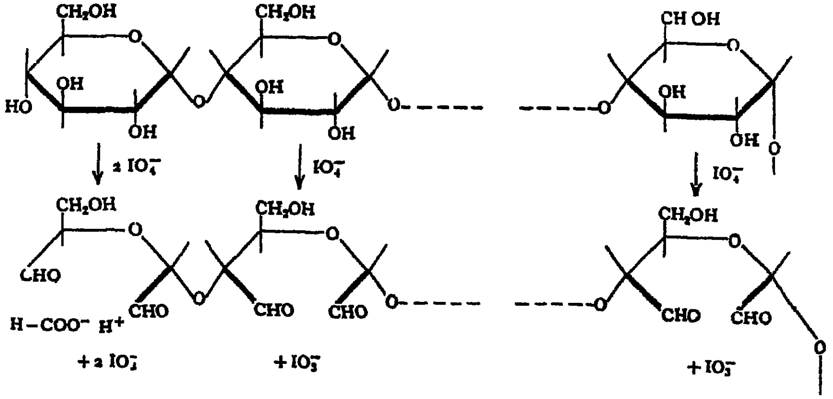
Branch-point radical. Yields a dimethylglucose.

Fig. 2. Diagrammatic representation of an 'atypical' unit chain.

It is also possible that the end-group of a unit chain might serve as the branch-point radical for *two* other chains. In this case this radical would give a molecule of dimethyl sugar in place of 2:3:4:6-tetramethylglucose.

The method of end-group assay was first developed by Haworth & Machemer (1932), who separated the methylated methylglucosides, obtained on methanolysis of methylated polysaccharides, by a process of fractional distillation under greatly reduced pressure. The fractionation was rendered more accurate by the observations of Hirst & Young (1938), and of Bacon, Baldwin & Bell (1944), but such procedures necessitate the handling of relatively large amounts of material for accurate results to be obtained, and by the distillation technique isolation of the homogeneous 'dimethyl fraction' (i.e. the branch-point radicals plus any artefacts) is extremely troublesome. Jones (1944) has devised a chromatographic micro method for the separation of the end-group as tetramethyl methylglucoside and Brown & Jones (1947) have further developed a small-scale partition method for the same purpose. Thus end-group assays can now be made on small amounts of material with a high degree of accuracy. Recently a method of specific oxidation of the end-groups of glycogens by periodate has been developed by Halsall, Hirst & Jones (1947).

This ingenious technique is based on the fact that of all the glucosyl radicals composing the glycogen molecule only those comprising the end-groups of the unit chains contain three hydroxyl groups in an unseparated series. Such a configuration yields, on oxidation by periodate ion, one molecule of formic acid as shown:



The formic acid is determined by a suitable procedure.

The results obtained by the above method, which requires only fractions of a gram of glycogen, show very close agreement with the findings from the methylation technique.

A successful procedure for the complete analysis of the hydrolysis products of methylated glycogen, as the free sugars, has been devised (Bell, 1944), applying to the series of tetramethyl-, trimethyl- and dimethylglucoses the principles of partition chromatography devised by Gordon, Martin and Synge. By this technique it is possible, by simple operations, to obtain complete separation of the different methylated fractions. As a result of these experiments, which required only one or two grams of material, it became clear that the dimethyl fraction was greater than was demanded by the theory expressed graphically in Fig. 1. As already stated, the failure to obtain genuinely fully methylated glycogen has defeated the aim for which this partition analysis is intended.

In Table 1 are set forth the known results of end-group assay experiments on different glycogens.

From these results, obtained by varied techniques, it will be apparent that glycogens can exist in chain-lengths averaging between 12 and 18 radicals.

It must be emphasized that chain-length determinations do not give information on the following questions: (1) whether, in a sample of glycogen every unit chain is exactly the same length or not; (2) whether all the branch-point radicals have the same structure or not, i.e. whether the interchain links are of the same type or not; (3) at what point in the unit chain branching occurs, i.e. whether it takes place at a definite distance from one or other end; (4) the size, or mass, of the glycogen molecules.

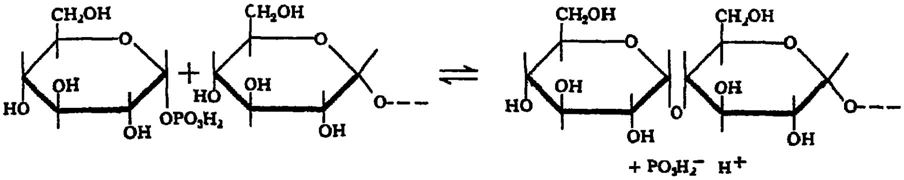
Table 1

Material	Unit chain length	Method of assay	Authors
Liver:			
Rabbit I	12	A	Haworth & Percival (1932)
Rabbit II	12	B	Bell (1935)
Rabbit II	12	C	Halsall, Hirst & Jones (1947)
Rabbit III	18	A	Haworth, Hirst & Isherwood (1937)
Rabbit III	16	C	Halsall *et al.* (1947)
Rabbit IV	18	C	Halsall *et al.* (1947)
Rabbit V and VI	18	B	Bell (1936*a*)
Rabbit VII	14	C	Halsall *et al.* (1947)
Rabbit VIII	18	E and C	Halsall *et al.* (1947)
Guinea-pig	13	C	Halsall *et al.* (1947)
Dog	12	B	Hassid & Chaikoff (1938)
Fish (Gadidae)	12	B	Bell (1935)
Fish (Haddock)	12	A	Haworth, Hirst & Smith (1939)
Fish (Hake)	12	A	Haworth, Hirst & Smith (1939)
Fish (Dogfish)	12	A	Haworth, Hirst & Smith (1939)
Muscle:			
Rabbit I	11	D	Bell (1948)
Rabbit I	12	C	Halsall *et al.* (1947)
Horse I	12	A	Bell (1937)
Horse I	12	D	Bell (1944)
Horse I	14	C	Halsall *et al.* (1947)
Man	11	C	Halsall *et al.* (1947)
Dogfish	12	A	Haworth *et al.* (1939)
Ascaris lumbricoides I	15–16	D	Bell (1944)
Ascaris lumbricoides I	13–14	A	Baldwin & King (1942)
Ascaris lumbricoides I	12	C	Halsall *et al.* (1947)
Helix pomatia	12	A	Baldwin & Bell (1940)
Mytilus edulis I	18	B	Bell (1936*b*)
Mytilus edulis II	10	A	Meyer & Fuld (1941)
Mytilus edulis III	10	C	Halsall *et al.* (1947)

A signifies fractional distillation of the methylglucosides (Haworth & Machemer, 1932).
B signifies large-scale partition (Bell, 1935).
C signifies periodate oxidation (Halsall *et al.* 1947).
D signifies small-scale partition (Bell, 1944).
E signifies partition by method of Brown & Jones (1947).

IV. THE BIOLOGICAL SYNTHESES OF GLYCOGEN AND THE QUESTION OF STRUCTURAL IDENTITIES

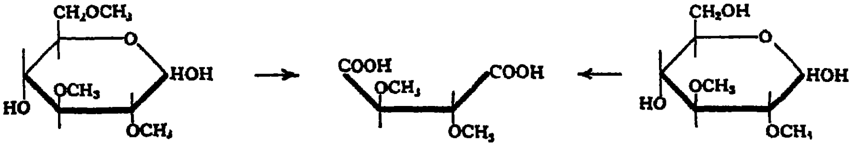
The discoveries of Cori and his collaborators, of Kiessling and of Ostern, Herbert and Holmes have left no doubt that the 1:4 α-glucosidic linkage between the radicals composing the unit chains of glycogen takes place through the synthetic action of phosphorylases. These enzymes perform the operation of exchanging the phosphoryl radical of α-glucose 1-phosphate for a glucosyl radical. The latter apparently must be present in an already sufficiently complex molecule, hence the necessity for 'priming' the process by addition of a small amount of glycogen, amylopectin or a dextrin derived from these two substances. The linkage formed is of the 1:4 type; the energy necessary to form the new link appears to be that already employed to bind the phosphoryl as an acid acetal or acid glucoside. This process of chain-building appears to belong to the now numerous class of radical transferring reactions such as transmethylation, transamidination, transphosphorylation, etc., many of which are freely reversible, as is indeed the reaction



Workers in Cori's laboratory have shown that the phosphorylase isolated from muscle tissue, despite the necessary priming, acting on α-glucose 1-phosphate forms an unbranched polysaccharide analogous to the amylose fraction of starch and staining a pure blue with iodine. Thus, this enzyme apparently synthesizes only the 1:4 linkage. On the other hand, phosphorylase preparations from liver and from yeast elaborate typical brown-staining polysaccharides which are assumed to have a branched structure. The muscle phosphorylase, however, when made to act in conjunction with a second catalyst present in muscle produces a typical red-brown staining material. This work has been admirably summarized by Hassid (1946). It is to be regretted that these synthetic products, excepting the 'muscle amylose' have not yet been subjected to chemical study. No doubt the recent inventions of micro methods for constitutional investigation of polysaccharides will be followed by reports on the structures of these substances.

It appears, therefore, that in muscle, at any rate, and possibly also in other situations of glycogen formation, we require two mechanisms at least in order to synthesize the branched polysaccharide. First, the 'phosphosynthetic' production of the unit chains, and secondly the formation, through the initiation of branching glucoside links, other than the 1:4 type, of the branched structure itself.

Whether there is any chemical distinction between glycogens of different origins remains to be proved. I am of the opinion, in view of my observations on molecular weights (see below), and on iodine colours, that differences may well exist, besides mere differences in molecular size. The methods of analysis which may lead to solving this problem have not yet been to any extent applied. I have attempted to analyse the composition of the 'branch-point fraction' obtained from methylated samples of rabbit liver and muscle glycogens (Bell, 1948). Owing, however, to the difficulty of deciding the origin of the dimethylglucoses obtained in this fraction, which is always greater in proportion than that of the end-group (see Fig. 1), these results were inconclusive in the case of the muscle polysaccharide. Definite evidence for the existence of a 1:3′ interchain link points to a difference between the branching of rabbit liver glycogen and branching of amylopectin. Some years ago, Haworth, Hirst & Isherwood (1937) examined the crude dimethyl methylglucoside fraction obtained during the large-scale fractional distillation of methanolysed rabbit liver glycogen. These authors stated, with reserve however, that they considered that the interchain linkage was possibly of the 1:6′ type. Their evidence was obtained by oxidation of their crude material with strong nitric acid whereby a small amount of dimethoxy succinic acid was produced presumably from 2:3-dimethylglucose. I consider this evidence to be inconclusive for the following reasons: (1) it is very difficult to eliminate, by distillation, the last traces of trimethyl methylglucoside from dimethyl methylglucoside; (2) during methanolysis, 2:3:6-trimethylglucose may undergo autocondensation to form a disaccharide which tends to contaminate the dimethyl methylglucoside fraction; (3) nitric acid oxidation of both 2:3:6-trimethylglucose and 2:3-dimethylglucose can produce the same dimethoxy succinic acid



2:3:6-Trimethylglucose Dimethoxy succinic acid 2:3-Dimethylglucose

It is unfortunate that this suggestion of the Birmingham workers has crept into many publications as a piece of concrete evidence.

It appears that our studies of glycogen structure have reached a stage where only enzymic degradations will help to clear up some of the present obscurities. Few investigations along such lines have been reported. Meyer & Fuld (1941) have shown that mussel glycogen is degraded by a maltogenic ('β') amylase to the extent of 47% of the chain-length (12 radicals). This indicates that the branch-point radical must be situated at a position more than six radicals from the end-group, thus defining a difference between amylopectin and this specimen of glycogen. No other application of this type of degradation to a glycogen of known chain-length has so far been reported.

A possible elucidation of the nature of the interchain link might well be afforded as a result of degradation of glycogen by a dextrinogenic ('α') amylase. By the action of such an enzyme from *Aspergillus oryzae*, on amylopectin, Montgomery, Weakley & Hilbert (1947) have isolated a 1:6 α-linked disaccharide which is apparently the branch-point radical with its interchain link intact. Such experiments applied to different glycogens would be of extreme interest and value.

V. MOLECULAR WEIGHTS OF GLYCOGENS

Numerous attempts have been made to determine the molecular weight of glycogen specimens, either on the free polysaccharide or on its acetyl or methyl derivatives. The methods normally used have been based on measurements of osmotic pressure or of viscosity. I believe that the only satisfactory method for use with a material so heterogeneous and of such high molecular weight as glycogen is one based on an actual estimation of mass. From sedimentation and diffusion constants, using the ultracentrifuge, it is possible to determine the *average* weight of the molecules comprising a sample of glycogen. By osmotic measurements on a polydisperse system of unknown composition it is only possible to determine the *number* of particles in solution (cf. Johnson, 1947). Table 2 gives measurements on glycogen samples which have been made, using sedimentation and diffusion data.

Table 2

Source	Mean molecular weight ($\times 10^6$)	Authors
Rabbit liver	4	Bridgeman (1942)
Rabbit liver	4·4–4·3	Bell, Gutfreund, Cecil & Ogston (1948)
Rabbit muscle	2·6	Bell *et al.* (1948)
Horse muscle	2·9	Bell *et al.* (1948)
Human muscle	2·4	Bell *et al.* (1948)
Ascaris tissue	0·7	Bell *et al.* (1948)
Mycobacterium tuberculosi	12–13	Chargaff & Moore (1944)

From the above results it will be seen that variations in molecular weights are considerable, according to the source from which the material was isolated. These molecules are among the largest soluble ones so far measured.

VI. SUMMARY

1. The usual criteria for identification of glycogen are discussed. It is pointed out that these can be applied equally well to amylopectin and α-amylodextrin. Numerous so-called glycogens in plants and micro-organisms may not be chemically identical with animal glycogens, which may quite well vary among themselves.
2. Mention is made of the methods of isolation and purification. It is considered that precipitation by acetic acid gives the purest material in that nitrogen and phosphate can be eliminated, in many cases entirely.
3. The limitations of the end-group assay are discussed. This method reveals the sole fact that all glycogens are built up from 'unit chains', but gives no information as to the detailed structure of these chains. It is, therefore, not possible to say whether glycogens from different sources are chemically the same or not.
4. A description is given of the difficulties in attempting to analyse glycogens from the aspect of determining the nature of the linkage uniting the unit chains. The suggestion is made that information to this end will have to be obtained by use of stepwise enzymic degradation of the polysaccharide.
5. The latest work on molecular weights is summarized. There is considerable variation according to the type of source from which the glycogen has been isolated. This work is the first indication that there are at least physical differences between glycogens.

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LE SYSTÈME HISTIOCYTAIRE OU RÉTICULO-ENDOTHÉLIAL

PAR M. CHÈVREMONT

Laboratoires d'Histologie et d'Embryologie, Université de Liège

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I. INTRODUCTION

Quand on injecte un animal, par exemple un lapin ou un cobaye, à l'aide d'un colorant non toxique, tel que le bleu trypan ou le bleu d'isamine, on remarque que sa peau se colore en bleu, en peu de jours. Si on le dissèque, on voit que certains organes ou tissus sont plus spécialement colorés (rate, conjonctif sous-cutané, ganglions lymphatiques, etc.). Cette coloration est dûe à la présence de nombreuses cellules possédant des caractères particuliers; ce sont les cellules histiocytaires, souvent encore appelées cellules réticulo-endothéliales. Depuis une cinquantaine d'années, elles ont été étudiées par de nombreux auteurs et une série de travaux ont démontré qu'elles jouent un rôle de premier plan à la fois dans les conditions normales et dans les conditions pathologiques.

Ces cellules sont capables d'absorber les éléments étrangers, vivants ou inertes. C'est ainsi qu'elles peuvent accumuler des particules de matières en suspension ou colloïdales introduites dans l'organisme par voie parentérale, telles que des colorants vitaux, de l'argent colloïdal, des particules de carbone (encre de Chine, etc.). Elles peuvent même phagocyter et éventuellement digérer des bactéries, des restes de cellules mortes ou des cellules usées ou étrangères. Beaucoup d'entre elles interviennent dans la formation des éléments figurés du sang (globules rouges, globules blancs). Elles prennent aussi une part active dans plusieurs métabolismes.

Si leur rôle est déjà remarquable dans les conditions normales, il est important aussi dans divers processus inflammatoires, localisés ou généralisés, aigus ou chroniques (comme la tuberculose, la lèpre) et en général dans les moyens de défense que le corps peut mettre en œuvre. Non seulement ces cellules interviennent activement dans la défense de l'organisme contre les éléments étrangers de diverses espèces ainsi que dans l'enlèvement des cellules détruites au cours de l'inflammation, mais elles peuvent aussi lutter contre les bactéries par la formation d'anticorps et contribuent à l'établissement de l'immunité. De plus, elles présentent le caractère remarquable de pouvoir se former sur place, aux dépens d'autres cellules, au cours de certaines circonstances. Enfin, alors que plusieurs de leurs propriétés peuvent rester latentes dans les conditions habituelles, elles peuvent s'actualiser et intervenir quand les conditions de milieu changent.

Ce qui contribue à leur conférer une importance particulière, c'est le fait qu'elles sont réparties à peu près dans tout l'organisme, soit comme cellules isolées, soit groupées en localisations déterminées. On peut dire, d'une manière schématique, qu'elles contrôlent et purifient le sang venant de l'intestin (au niveau du foie) et le sang en général (directement dans le sang lui-même par le monocyte ainsi que dans la rate et la moelle osseuse). Elles contrôlent également la lymphe périphérique et intestinale (au niveau de diverses formations lymphatiques). Elles sont en général présentes en grand nombre dans le tissu conjonctif de la peau et des organes, dans certaines glandes endocrines et vraisemblablement dans les poumons.

Ces cellules sont mobiles ou susceptibles de le devenir. D'une manière générale, on peut en reconnaître deux types principaux: les histiocytes et les macrophages. Tandis que les premiers correspondent plutôt à une forme de repos, quiescente, les seconds représentent des éléments très actifs et mobiles, ayant souvent phagocyté des éléments volumineux. Histiocytes et macrophages ont été décrits en détail, notamment par Maximow (1927, 1928) et par Levi (1934).

Malgré leur dispersion et malgré certaines dissemblances structurales et fonctionnelles, ces cellules sont essentiellement de la même nature et forment en réalité un groupe cellulaire particulier, un vaste système qui est appelé le système réticulo-endothélial (Aschoff, 1924) ou histiocytaire. Ce concept repose sur une base fonctionnelle beaucoup plus que sur une base structurale.

Enfin, du point de vue pathologique, il faut remarquer que les cellules appartenant à ce système peuvent présenter des maladies particulières, qui sont plus ou moins diffuses ou systématisées, ou portent parfois sur un secteur de ce système. On connait aussi des tumeurs 'réticulo-endothéliales'.

Les cellules histiocytaires ou réticulo-endothéliales représentent donc un des éléments fondamentaux des animaux supérieurs, à la fois par leur masse totale et par leurs caractères biologiques. Elles apparaissent très tôt au cours du développement embryonnaire. Elles existent chez tous les Vertébrés et on trouve aussi des cellules analogues chez beaucoup d'Invertébrés. Diverses recherches récentes de biologie cellulaire ont permis, d'une part, de pénétrer plus profondément dans leur connaissance et, d'autre part, de modifier des conceptions classiques ou de dégager des conceptions nouvelles. Nombreuses sont en outre les perspectives d'avenir qui s'ouvrent dans ce domaine.

II. CARACTÈRES CYTOLOGIQUES ET PROPRIÉTÉS BIOLOGIQUES DES CELLULES HISTIOCYTAIRES

Les cellules réticulo-endothéliales, ou mieux histiocytaires, se distinguent par un ensemble remarquable de caractères cytologiques et de propriétés biologiques. Rappelons ici leurs caractères essentiels, en y ajoutant les connaissances qui ont été acquises plus récemment.

Les *histiocytes*, qui correspondent à un stade d'activité cellulaire moindre, sont des cellules mobiles ou susceptibles de le devenir. Quand ils se déplacent, c'est à l'aide de pseudopodes. Le plus souvent ils sont allongés et munis de prolongements cytoplasmiques parfois ramifiés. Ils ne contiennent que peu d'enclaves figurées. Ce sont les 'resting wandering cells' décrites par Maximow (1906, 1928).

Les *macrophages* sont des cellules libres, arrondies ou à peu près. Ils sont caractérisés par la présence d'une membrane ondulante très mince et transparente, avec des plis; c'est grâce à elle qu'ils sont très mobiles. Ils renferment de nombreuses vacuoles colorables par le rouge neutre, quelques gouttelettes lipidiques, ainsi que, fréquemment, du matériel phagocyté. Ce sont les 'amoeboid wandering cells' de Maximow.

Toutefois, histiocytes et macrophages sont essentiellement de la même nature et possèdent les mêmes potentialités et les mêmes propriétés biologiques. On peut trouver entre eux tous les intermédiaires et ils se changent facilement l'un en l'autre, selon les conditions du milieu. Cette distinction en deux grandes catégories est admise par de nombreux auteurs (Maximow, Ephrussi, Chèvremont, etc.) mais pas par d'autres. C'est ainsi que Thomas (1938) reconnaît plutôt l'existence de trois aspects de cellules histiocytaires: le macrophage typique arrondi; la cellule allongée munie de prolongements minces, parfois ramifiés, au bout desquels apparaît un voile hyaloplasmique; enfin, la cellule arrondie ou faiblement allongée, pourvue d'une membrane ondulante, mais émettant un ou quelques prolongements courts. Nous préférons la première classification parce qu'elle est bien représentative de deux états cellulaires, entre lesquels existent toutefois plusieurs intermédiaires. Parmi les caractères qui permettent de reconnaître les macrophages, la membrane ondulante* présente une importance particulière. Changeant continuellement de forme et d'étendue, elle varie aussi dans le temps et peut disparaître pour redevenir visible en un autre endroit de la surface cellulaire.

Existe-t-il des différences structurales entre les cellules histiocytaires des différentes localisations? Il y a entre elles de légères différences, comme Maximow l'a déjà signalé (1928). Ainsi les histiocytes du tissu réticulé de la rate sont souvent munis de prolongements cytoplasmiques plus longs que ceux des histiocytes du tissu conjonctif lâche (appelés autrefois clasmatocytes). Ces derniers histiocytes ont un aspect différent de celui d'histiocytes plongés dans un milieu liquide, les monocytes du sang. Alors que dans le conjonctif lâche, dans le sang, les cellules histiocytaires sont séparées, elles sont au contraire groupées et se touchent souvent par leurs prolongements dans les organes hématopoïétiques. J'ai récemment individualisé et décrit un type particulier d'histiocyte: les *histiocytes graisseux* (Chèvremont, 1942).

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* Ce terme de membrane ondulante doit être adopté, quoiqu'il ne s'agisse pas d'une véritable membrane ni d'un organe cellulaire permanent comme c'est le cas chez certains Hématozoaires. Il doit être employé comme une expression imagée (Policard, 1926). La membrane ondulante a été appelée parfois aussi 'hyaloplasme' (Renaut, 1907) ou 'exoplasme' (Carrel et Ebeling, 1926). On sait que Carrel (1926) en a fait une étude cinématographique, en partie avec un éclairage latéral. Elle existe aussi dans les choanoleucocytes ou amibocytes actifs des Invertébrés, qui se rapprochent des macrophages (Fauré-Fremiet, 1925–9; Lison, 1930).

L'origine et la nature des cellules adipeuses ont été discutées depuis longtemps (Flemming, Toldt, Hammar, Maximow, Clara, E.R. et E. L. Clark, et d'autres). On sait qu'il faut distinguer, surtout au point de vue de leur origine et de leur développement, la graisse de formation primaire et celle de formation secondaire. Wassermann (1925–9), Dogliotti (1928) et Godina (1938) principalement ont montré que la graisse primaire est de nature histiocytaire. Quant à la question de l'origine de la graisse secondaire, j'ai apporté une contribution à sa solution en montrant qu'il existe dans le tissu sous-cutané (embryons de poule et poussins) des histiocytes particuliers d'ou dérivent les cellules adipeuses de la graisse secondaire. Ce problème a été abordé à la fois par la méthode de la coloration post-vitale (examen de fragments minces de sous-cutané, laissés en contact avec une solution de rouge neutre), par la méthode des cultures *in vitro* et enfin par les techniques histochimiques des lipides. Ces histiocytes graisseux présentent un aspect caractéristique: ce sont des cellules assez aplaties généralement sans prolongements, qui sont chargées de nombreuses petites gouttelettes lipidiques. Les lipides apparaissent dans des vacuoles colorables par le rouge neutre; puis au fur et à mesure qu'ils se déposent, la colorabilité par le rouge neutre diminue et disparaît. Ces petites gouttelettes lipidiques font peu à peu place à quelques grosses gouttes, et la cellule prend progressivement l'aspect d'une cellule adipeuse typique, à volumineuse goutte lipidique unique. Fait remarquable, les caractères histochimiques de ces lipides changent en même temps que leur aspect. Tout d'abord ce sont presqu'exclusivement des lécithines, puis en passant par une série d'intermédiaires, les lipides deviennent surtout des glycérides, avec un petit pourcentage de cholestérides et de cholestérol, et très peu ou pas de lécithines (ce qui correspond aux résultats fournis par l'analyse chimique sur un lobule de graisse correspondant).

Quant à leur origine, on peut supposer que ces histiocytes graisseux dérivent de cellules mésenchymatiques ou conjonctives. Cette manière de voir permet aussi de comprendre comment n'importe quel fibrocyte peut devenir une cellule adipeuse, au cours de l'engraissement ou de l'obésité (Schaffer, 1930). Dans cette circonstance, les fibrocytes auraient la capacité de se transformer en histiocytes graisseux, comme ils peuvent dans d'autres conditions devenir des histiocytes ou des macrophages.

Du point de vue fonctionnel ou évolutif, il peut exister des différences entre histiocytes de diverses localisations. On sait par exemple que la potentialité hématopoïétique s'actualise davantage dans un certain sens au niveau des cellules réticulées des formations lymphatiques, dans un autre sens au niveau de celles de la moelle osseuse hématopoïétique, etc.; un hémocytoblaste peut évoluer en hématie, granulocyte ou mégacaryocyte. Quelle raison explique la différenciation de la cellule-souche dans l'un ou l'autre sens? Nous l'ignorons encore. On peut supposer qu'il s'agit d'un mécanisme chimique. Ce dernier pourrait être analogue à celui que nous signalerons plus loin à propos du déterminisme réglant la formation des cellules histiocytaires. Peut-être seraient-ce des substances diverses, ou plus ou moins voisines, qui orientent l'évolution cellulaire dans l'une ou l'autre direction. Il serait d'un grand intérêt d'être fixé sur ces points.

Dans certains cas on trouve aussi des différences entre histiocytes d'un type donné. Tout récemment, Lison (1947 et communication personnelle) a en effet montré qu'il y a deux types de cellules de Kupffer, les unes athrocytant les colorants à petites particules, les autres ceux à grosses particules. Tandis que chez les Amphibiens les cellules des deux types sont réparties au hasard, elles se localisent de façon assez régulière chez les Mammifères (rat): les athrocytes prenant les grosses particules sont surtout situés à la périphérie du lobule et les autres sont répartis assez également partout.

Nous venons de voir qu'il existe quelques différences structurales entre les histiocytes des diverses localisations. Au contraire, l'aspect des macrophages varie surtout d'après les conditions d'étalement, le nombre et les dimensions de leurs enclaves lipidiques ainsi que la quantité du matériel phagocyté. Dans de nombreux cas étudiés ils n'ont montré entre eux aucune différence structurale qui soit en relation avec leur origine ou leur distribution. Par exemple, les macrophages dérivés des monocytes sont identiques à ceux des tissus (Carrel & Ebeling, 1926). Signalons cependant un cas où des différences se marquent en relation avec leur origine. Ainsi, un facteur peut influencer la taille des macrophages, c'est le volume des cellules dont ils proviennent dans certains cas (voir ci-après la transformation histiocytaire). En effet, quand un macrophage provient d'un élément volumineux (musculaire, par exemple), il est plus grand que celui qui dérive d'éléments plus petits* (Chèvremont, 1942).

Du point de vue biologique, les macrophages présentent les mêmes propriétés quelles que soient leur origine et leur distribution. On connait pourtant un cas où des macrophages résultant d'une transformation histiocytaire pourraient conserver des caractères fonctionnels des cellules dont ils dérivent. C'est celui des macrophages provenant de la transformation de cellules entoblasto-vitellines, qui peuvent *in vitro* continuer à sécréter un produit comparable au vitellus (Thomas, 1938).

Actuellement, il est bien connu que les cellules histiocytaires peuvent aisément prendre l'aspect de cellules épithélioïdes ou de cellules géantes, lesquelles sont également de nature histiocytaire. Tandis que les premières sont aplaties, à contours faiblement silhouettés, souvent groupées et ressemblant à des cellules épithéliales, les secondes sont de volumineux éléments renfermant souvent dix à vingt noyaux situés au centre. Ces cellules jouent un rôle important dans des réactions cellulaires contre des éléments étrangers et dans certains phénomènes pathologiques, comme la tuberculose.

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* L'influence de ce facteur apparaît clairement dans une même culture de tissu où les éléments cellulaires vivent donc dans des conditions de milieu aussi semblables que possible. De deux macrophages, d'un même type assez étalé, celui qui provient d'un élément plus volumineux mesure, par exemple, 87 μ de long et 50 μ de large, tandis que l'autre mesure respectivement 37 μ et 16 μ.

La grande diversité d'aspects des cellules histiocytaires, qui vont de l'histiocyte allongé ou du macrophage arrondi et pourvu d'une membrane ondulante à la cellule épithélioïde et à la cellule géante, n'est que l'expression de la *plasticité* étonnante de ces cellules, un de leurs caractères les plus frappants. Comme le souligne Carrel, chaque type cellulaire est identifiable aussi bien par ses propriétés fonctionnelles que par son aspect morphologique, et c'est particulièrement vrai dans le cas des cellules histiocytaires. Les cellules histiocytaires sont remarquables, en effet, non seulement par leur aspect cytologique mais aussi par une série de propriétés biologiques. Elles se caractérisent notamment par leur *mobilité*, spécialement les macrophages. Entrant en contact avec une surface solide, ils y adhèrent grâce à leur membrane ondulante; ils ont ainsi la tendance bien connue à se placer aux interfaces et à s'arrêter au contact de corps étrangers.

Une étude histochimique approfondie des gouttelettes lipidiques visibles dans les cellules histiocytaires révèle, dans l'ensemble, qu'elle scontiennent surtout des lécithines, très peu de glycérides et souvent, avec l'âge, une petite quantité de cholestérides (Chèvremont, 1942).

Les cellules histiocytaires possèdent un pouvoir très élevé de se colorer par les colorants vitaux basiques ou acides, ainsi que de phagocyter (des débris cellulaires, globules rouges, etc.). En particulier, elles peuvent accumuler dans leur cytoplasme, sous forme figurée et généralement granulaire, des substances diverses, dissoutes ou en suspension fine qui arrivent à leur contact; c'est le pouvoir *d'athrocytose* (Gérard & Cordier 1934), ou 'Speicherung' des auteurs allemands, qui ne se rencontre, chez les Vertébrés, que dans les cellules histiocytaires et les tubes contournés du rein.

Rappelons à ce propos qu'il ne suffit pas de constater simplement que des cellules accumulent du colorant vital ou encore phagocytent pour être autorisé à conclure que les cellules en question sont réticulo-endothéliales ou le sont devenues. A la suite d'une surcharge massive en bleu trypan (injecté expérimentalement), par exemple, il peut se faire que de simples fibrocytes de la capsule des ganglions lymphatiques renferment aussi du colorant vital, quoiqu'à un degré moindre que les véritables cellules histiocytaires voisines. De même, de la seule *phagocytose* par une cellule on ne peut conclure à la nature histiocytaire de celle-ci. Fischer & Laser (1927), notamment, ont pu provoquer la phagocytose dans les fibrocytes en culture par l'addition, au milieu, d'oléate de soude ou d'uréthane. Il s'agit ici d'une simple incitation à la phagocytose. Pour pouvoir admettre la nature histiocytaire d'un élément, il ne suffit donc pas d'observer chez lui de l'athrocytose ou bien de la phagocytose, il faut vérifier un ensemble de plusieurs caractères et propriétés démonstratifs. Faute de quoi, on risque d'aboutir à des conclusions erronées, comme le font encore certains auteurs.

Les macrophages peuvent également absorber des gouttelettes lipidiques dans leur cytoplasme (Lewis, 1931). Ce phénomène s'appelle la *pinocytose* et n'est pas tout-à-fait spécifique des macrophages puisqu'il a aussi été observé dans des cellules cancéreuses *in vitro*.

Les cellules histiocytaires possèdent encore d'autres propriétés importantes, qui ont été signalées depuis plusieurs années et que nous n'exposerons pas en détail ici. Elles sont aussi plus *résistantes* que d'autres cellules à des substances comme l'arsenic, ou encore, selon Lasfargues (1946), à des exotoxines microbiennes (exotoxine staphylococcique, par exemple). Elles peuvent aussi, comme Carrel & Ebeling (1922*a*, *b*) l'ont démontré pour les monocytes, se nourrir *in vitro* uniquement aux dépens des constituants protidiques du plasma ou du sérum, ce que ne peut faire aucune autre race cellulaire. En outre, elles peuvent rendre ce milieu viable pour les fibroblastes, en transformant les protides du sérum en une forme utilisable par eux. D'une manière générale, en conditions pathologiques ou non, elles résistent beaucoup mieux que d'autres cellules dans les milieux déficients en matériaux nutritifs et en oxygène.

Une grande richesse en *ferments protéolytiques* et en *lipases* caractérise également les cellules histiocytaires. C'est grâce à ces ferments qu'elles transforment et digèrent plus ou moins, selon sa nature, le matériel qu'elles ont phagocyté.

Un autre rôle de premier plan est encore joué dans la *dégradation de l'hémoglobine* par les cellules histiocytaires (du foie, de la moelle osseuse et particulièrement de la rate).

Enfin, diverses cellules histiocytaires possèdent aussi un *pouvoir hématopoïétique* caractéristique, auquel nous avons déjà fait allusion plus haut. Dans certaines conditions des phénomènes de vicariance peuvent se produire, et des cellules histiocytaires peuvent manifester alors telle capacité hématopoïétique qu'elles ne réaliseraient pas normalement.

Un rôle considérable est aussi joué par les éléments du système histiocytaire dans *divers métabolismes*, ceux du fer, des lipides, du cholestérol, des vitamines. Quand on donne, par exemple, de la vitamine A à un animal, c'est surtout dans le cytoplasme des cellules histiocytaires qu'elle se retrouve, comme on peut le voir à l'aide du microscope à fluorescence (Wimmer, 1939). On peut aussi observer *in vitro* que les macrophages sont capables d'absorber de la vitamine A ajoutée aux cultures (Chèvremont, 1942). La vitamine D est aussi spécialement retenue par les cellules histiocytaires (Pittaluga, 1936).

Les cellules histiocytaires possèdent aussi à un haut degré le pouvoir d'absorber la vitamine C. Les méthodes histochimiques ont permis de la déceler *in situ*, parmi d'autres cellules de l'organisme, dans les monocytes (Giroud, 1934), les histiocytes du tissu conjonctif (Giroud, 1938; Tonutti, 1940, et d'autres), les cellules du réticulum des organes hématopoïétiques (Hammar, 1938; Tonutti, 1940), les cellules de l'endothélium sinusoïde (Giroud, 1938), les phagocytes alvéolaires du poumon (Tonutti, 1940). La richesse des macrophages en vitamine C a encore été observée dans l'organisme lors de la résorption de fils de catgut, de fibres musculaires dégénérées (Klein, 1938). Si on injecte de la vitamine C à un animal, c'est dans les cellules histiocytaires qu'on en retrouve une large part. Wimmer (1939) a insisté sur le rôle qu'elles jouaient dans le métabolisme de cette vitamine.

Par la méthode de la culture des tissus, il a été démontré récemment que les cellules histiocytaires possèdent un pouvoir remarquable d'absorber des quantités élevées d'acide ascorbique. Elles peuvent en absorber des quantités nettement plus considérables que d'autres cellules (fibrocytes, éléments musculaires, cellules endothéliales, épithéliales, etc.) (Chèvremont, 1942). Déjà 15 minutes après l'addition d'acide ascorbique aux cultures, les résultats histochimiques (méthode de Giroud) sont nets et le maximum est obtenu après 2 heures 30 environ. On trouve de nombreux grains d'argent réduit dans les cellules histiocytaires, peu dans les autres cellules. Ce fait est important parce qu'il éclaire le rôle que peuvent jouer les cellules histiocytaires et conduit à penser qu'elles doivent une partie de leurs propriétés à la vitamine C qu'elles peuvent absorber.

L'importance de l'acide ascorbique en histopathologie a été soulignée par les observations de Tonutti et ses collaborateurs (1938–40). Ces auteurs ont montré l'abondance de la vitamine C dans certaines cellules histiocytaires au cours de la pneumonie (Tonutti & Matzner, 1938) et de la tuberculose (Tonutti & Walraff, 1939). Dans ce dernier cas, les phagocytes actifs des tubercules contiennent des quantités très considérables de vitamine C; leur teneur est même plusieurs fois supérieure à celle des capsules surrénales; on peut l'estimer approximativement à 300 mg. pour 100 g.; or, ces concentrations paraissent suffisantes pour inhiber le développement de divers microbes pathogènes ou même pour les détruire. On est autorisé, semble-t-il, à attribuer, avec Tonutti & Matzner, avec Klein (1938), l'activité fermentaire des histiocytes au point de vue de la défense de l'organisme à la vitamine C. Sa présence permet peut-être aussi d'expliquer leur grande capacité protéolytique.

Dans l'emmagasinage et le transport des produits chimiothérapiques, dont l'activité est bien connue en thérapeutique humaine, les cellules histiocytaires interviendraient aussi activement (Aschoff et son école).

Enfin, de nombreux auteurs ont insisté sur l'importance qu'elles présentent dans la *défense de l'organisme* contre l'infection et dans l'immunité, processus qui sont intimement liés à leur pouvoir d'athrocyter et de phagocyter, ainsi qu'à leurs ferments.

Aspect cytologique, mobilité, plasticité marquée, richesse en ferments, glycolyse élevée, pouvoir considérable d'athrocytose et de phagocytose, capacité particulière d'absorber des vitamines, résistance et exigences nutritives particulières, rôle important dans divers métabolismes et dans la défense de l'organisme, etc., tels sont donc les caractères des cellules histiocytaires. Ce qui augmente encore leur importance particulière, c'est le fait que, munies de telles propriétés, elles sont réparties à peu près dans tout l'organisme. Si chaque cellule histiocytaire, vivant dans des conditions données, n'exerce pas à tout moment ses différentes capacités, celles-ci peuvent, en principe, s'actualiser et intervenir lorsque le milieu se modifie et que les conditions changent.

Voyons en résumé quelle est la *localisation* des cellules histiocytaires dans l'organisme. On peut distinguer: (1) les histiocytes proprement dits, situés dans le tissu conjonctif, en particulier dans le conjonctif lâche; (2) les monocytes du sang, qui peuvent sortir des vaisseaux et s'installer dans les tissus, ou inversement; (3) l'endothélium sinusoïde veineux, qui se rencontre dans la rate et la moelle osseuse; (4) les cellules réticulées de la rate, de la moelle osseuse et des organes lymphatiques; (5) les cellules de Kupffer, ancrées aux parois des capillaires discontinus du foie et distinctes de leur endothélium; (6) les histiocytes graisseux; (7) les cellules adventitielles des vaisseaux, certains péricytes.

L'existence de cellules histiocytaires a encore été envisagée par une série d'auteurs dans diverses localisations, notamment dans le poumon (cellules alvéolaires) et dans le système nerveux (microglie). Si ces cellules ne sont pas de nature histiocytaire, au moins peuvent-elles très facilement passer à l'"état histiocytaire' sous l'effet de certaines incitations.

Dans les circonstances habituelles, les macrophages sont beaucoup moins nombreux que les histiocytes, mais le nombre des premiers peut augmenter considérablement, au cours de l'inflammation par exemple. Même quand elles sont fixées ou disposées en tissus, les cellules histiocytaires peuvent toutes devenir des macrophages. Après avoir athrocyté ou phagocyté, elles peuvent se séparer de leurs voisines, s'arrondir et se mobiliser. De plus, les cellules histiocytaires peuvent aussi *se former sur place*, aux dépens de différentes cellules, comme nous le verrons plus loin.

III. LA TRANSFORMATION HISTIOCYTAIRE *IN VITRO*

Il est remarquable que les cellules histiocytaires peuvent en effet être formées *de novo*, à partir de cellules d'un tout autre type; c'est la transformation histiocytaire. L'existence d'un tel processus a été établie *in vivo* au cours de l'inflammation et *in vitro* dans les cultures de tissus. Considérons tout d'abord ce qui se passe *in vitro*; nous reviendrons plus loin sur la façon dont cette question se présente *in vivo*.

Les cellules histiocytaires peuvent dériver de lymphocytes (Maximow, 1925; Bloom, 1931, etc.), de fibrocytes appartenant à des souches de cœur, ou de tissu conjonctif (Carrel & Ebeling, 1926; Fischer, 1927; de Haan, 1927; W. et M. von Möllendorff, 1929–32; Ephrussi & Hughes, 1930; Parker, 1932; Thomas, 1934–8, etc.). La possibilité d'une transformation histiocytaire a aussi été démontrée *in vitro* pour les cellules d'autres tissus: le mésenchyme (Fauré-Fremiet & Garrault, 1932, etc.), l'entoblaste vitellin de la vésicule ombilicale (Thomas, 1935, 1936, 1938), le mésenchyme et le tissu conjonctif sous-cutané à différents âges, les muscles squelettiques, les myotomes, les muscles ciliaire et de l'iris et les fibres musculaires lisses (amnios) (Chèvremont, 1940, 1942).

Dans les cultures de muscles squelettiques ou de tissu conjonctif sous-cutané, le processus transformateur se produit spontanément et avec une grande fréquence, contrairement à ce qui avait été observé par différents auteurs pour les 'fibrocytes' de souches transplantées de nombreuses fois (Chèvremont, 1942). Les faits sont particulièrement suggestifs dans les cultures *in vitro* de muscles squelettiques. On y observe des 'formes de transition' bien reconnaissables et le processus évolutif peut être suivi de bout en bout dans les cultures vivantes, maintenues à 38°. La transformation histiocytaire se produit à partir d'éléments de nature musculaire certaine: cellules musculaires isolées, bourgeons plurinucléés ou fragments plasmodiaux. Voici, en résumé, comment elle se présente.

Dans le premier cas, des cellules musculaires entières montrent une transformation fondamentale de leur aspect, de leurs propriétés et de leur nature. Ces modifications portent à la fois sur la forme générale, le noyau, la substance fondamentale du cytoplasme, les chondriosomes et les enclaves paraplasmiques. Des gouttelettes lipidiques apparaissent vraisemblablement par libération ou désintégration de cénapses lipoprotidiques. En même temps, le nombre des grains et des vacuoles colorables par le Rouge neutre augmente. La cellule acquiert progressivement le pouvoir d'athrocyter et de phagocyter, se munit d'une membrane ondulante, bref prend tous les caractères cytologiques et biologiques d'un macrophage. A 38° cette transformation histiocytaire est fréquente et se produit en 4 à 6 heures.

Au niveau des bourgeons musculaires caractéristiques, la transformation se fait selon deux processus différents: tantôt une cellule s'individualise et se libère du bourgeon, puis elle se transforme en macrophage; tantôt, une partie même du bourgeon se transforme directement. Quand il s'agit de fragments musculaires plasmodiaux, à noyaux peu nombreux, une partie du territoire donne souvent naissance à un macrophage. On observe ainsi, après l'apparition d'une membrane ondulante, des images de transition très démonstratives, par exemple d'une formation cellulaire, à deux noyaux, dont une moitié présente un aspect musculaire net tandis que l'autre a incontestablement acquis un type histiocytaire, alors qu'il y a toujours continuité entre les deux, sans limite visible.

Récemment aussi, Weiss (1944) a montré que dans les cultures de fragments de nerfs périphériques ou de ganglions spinaux, des cellules de Schwann et des fibroblastes se transforment en macrophages; beaucoup des cellules transformées sont géantes et plurinucléées. Ce processus se produit régulièrement.

IV. PRODUCTION EXPÉRIMENTALE DE LA TRANSFORMATION HISTIOCYTAIRE *IN VITRO*

Depuis une quinzaine d'années, plusieurs chercheurs se sont attachés à provoquer expérimentalement la transformation histiocytaire, c'est-à-dire le changement de différentes cellules en histiocytes et macrophages. Presqu'exclusivement dans les cultures *in vitro* et le plus souvent dans des souches de fibrocytes, ils ont utilisé, avec des succès divers, des moyens variés, tels que irritations, infections et intoxications des cellules.

C'est ainsi qu'ont été employés la tuberculine (Fischer, 1927), des infections (de Haan, 1927), des autolysats de bacilles typhiques ou leur toxine (Spadafina, 1933). Dans quelques autres cas, il s'agit d'irritations, fortes (de Haan, 1927) ou légères et chroniques (arsenic) (M. von Möllendorff, 1929, 1931), ou de l'extrait concentré de leucocytes (de Haan, 1927), ou encore un colorant tel que le bleu trypan, accompagné d'extrait de rate (M. von Möllendorff, 1931). Parfois les auteurs ont eu recours à la résection d'un secteur de la culture (Ephrussi, 1930), à un changement de pH (Feringa & de Haan, 1934), au chauffage dans de l'hydrogène (M. von Möllendorff, 1931), ou au lavage des cultures avec du liquide de Ringer oxygéné et chauffé (Kokott, 1931), à la culture en plasma hépariné (Parker, 1932), ou, enfin, à la culture prolongée sans lavage ni transplantation (Szantroch, 1932), etc.

Ces résultats ont été vivement critiqués par Demuth (1934) et surtout par Fischer-Wasels (1929) et par Tannenberg (1930–1). Ces auteurs n'admettent pas que les fibroblastes puissent se transformer en macrophages, comme le pensaient Carrel & Ebeling, Fischer, W. et M. von Möllendorff, Schultz, etc. Pour Tannenberg (1930), les formes arrondies observées dans les cultures étaient des formes dégénératives de fibrocytes et non des cellules histiocytaires.

Si certaines observations de production expérimentale de la transformation histiocytaire sont douteuses ou ont été établies sur des bases insuffisantes, d'autres sont définitivement acquises. Pour être valables, il est nécessaire qu'elles soient basées sur l'étude prolongée des cellules vivantes et sur la vérification à la fois des caractères cytologiques et des propriétés biologiques des macrophages nouvellement formés. Il faut, en effet, vérifier tous les critères requis. C'est ainsi que la seule accumulation de colorants vitaux ou la seule phagocytose ne correspond pas nécessairement à une véritable transformation histiocytaire, comme nous l'avons déjà dit.

Un petit nombre d'auteurs ont réussi à causer une véritable transformation histiocytaire par des substances chimiques pures.

M. von Möllendorff (1931) et Spadafina (1933) ont obtenu un tel effet respectivement avec l'arsenic ou le sulfate d'atropine. Tandis que la première a employé les fibrocytes de souches stabilisées de lapin adulte, le second a utilisé des cultures de cœur ou de mésenchyme des gros vaisseaux de la base du cœur. Mais c'est plutôt à une intoxication cellulaire légère que l'action de ces substances semble être attribuable.

Thomas (1935, 1936, 1938) a obtenu d'intéressants résultats dans ses recherches sur l'entoblaste vitellin de la vésicule ombilicale. La transformation de ces cellules en histiocytes, qui se produit spontanément dans 3 % des cas, devient plus fréquente si les cultures sont traitées par NaOH ou KOH à *M*/100, dans des conditions définies; il y a respectivement 32 et 12 % de transformation (en pour cent de cultures en flacon Carrel). De son côté, le liquide provenant de cultures ainsi traitées fait apparaître des histiocytes dans 53 % de nouvelles cultures. Ensuite, Thomas a essayé différents produits avec des résultats variables (acides, baryte, extraits de vitellus, oléate de soude, cholestérol, venin de cobra, lysocithine, etc.). Les ammoniums quaternaires du groupe de la choline se sont montrés actifs sur la transformation de cellules vitellines en histiocytes, particulièrement le triméthyléthylammonium, qui produit 59 % de transformation. L'auteur pense (1936, p. 40) qu'il est possible que, dans toutes ses premières expériences à résultats positifs, l'hydrolyse des lécithines du vitellus des cellules vitellines ait abouti à la libération d'ammoniums quaternaires dans le cytoplasme. Ceux-ci ont contribué à déclencher la transformation histiocytaire de certaines cellules vitellines.

De mon côté, j'ai recherché (Chèvremont, 1943 *b*, *c*) si la transformation histiocytaire peut être influencée dans les cultures *in vitro* par divers corps purs, chimiquement définis qui sont importants au point de vue de la croissance et du métabolisme cellulaire, et qui sont en même temps des constituants normaux des tissus ou peuvent dériver de ceux-ci (histidine, nucléinate de soude, acétylcholine, adrénaline, etc.). Ces expériences réalisées sur des cultures de muscles squelettiques ('petits explants') et parfois de tissu conjonctif sous-cutané, ont démontré notamment que la choline, ajoutée expérimentalement aux cultures, favorise la transformation histiocytaire, d'une manière tout-à-fait remarquable. Dans les cultures témoins, les cellules ne se changent que rarement en macrophages, mais si on ajoute de la choline aux cultures de muscles squelettiques (à la concentration optima de *M*/300 pour les cultures en goutte pendante ou de *M*/750 pour les flacons), le nombre des macrophages s'élève respectivement à près de 300% et plus de 450% de celui des cultures témoins. La choline détermine aussi de très nombreuses cellules conjonctives à se transformer en macrophages. L'acétylcholine a une action analogue à celle de la choline, mais légèrement moins forte.

V. DÉTERMINISME DE LA TRANSFORMATION HISTIOCYTAIRE *IN VITRO*

Des travaux qui viennent d'être résumés il ressort qu'un petit nombre de corps chimiques à formule définie peuvent provoquer expérimentalement la transformation histiocytaire. C'est le cas pour la choline ou pour des corps voisins de celle-ci et à activité semblable (expériences réalisées *in vitro* sur les cellules entoblasto-vitellines par J. A. Thomas (1935–8), sur les éléments musculaires et les cellules conjonctives par Chèvremont (1943 *b*, *c*). Si elles permettent d'envisager la possibilité de l'action de la choline ou d'autres ammoniums quaternaires de son groupe, les observations citées plus haut ne démontrent pas quels facteurs interviennent effectivement dans la transformation histiocytaire qui se produit en dehors de toute addition de substances étrangères.

C'est à l'étude de cet important problème du déterminisme de la transformation histiocytaire que je me suis attaché de 1941 à 1945, seul ou avec des collaborateurs (S. Chèvremont-Comhaire, Bacq). Ces expériences ont été réalisées sur des cultures *in vitro* de muscles squelettiques et de tissu conjonctif sous-cutané n'ayant subi aucun repiquage, sauf dans un cas. Ces cultures sont particulièrement favorables à la fois par la clarté de leurs aspects cytologiques et par l'énorme variation de fréquence qu'on peut produire dans la transformation histiocytaire en changeant les conditions expérimentales. En outre, leurs cellules sont à un état biologique très semblable à celui qu'elles présentent dans l'organisme.

Comme nous l'avons déjà dit, lorsqu'on cultive des fragments de muscles squelettiques, par exemple, atteignant des dimensions convenables, on est surpris par le nombre élevé de macrophages authentiques qui apparaissent spontanément dans ces cultures. Dans ce phénomène, le rôle déterminant n'est pas joué par le plasma, la quantité de milieu nutritif, la réduction de la tension d'oxygène, une modification de pH; toutefois l'extrait embryonnaire exerce une action favorisante (Chèvremont, 1942, chap. 3).

Un facteur tissulaire s'est rapidement révélé comme important: c'est le volume des explants ou la quantité de tissus. En effet, quand les explants ont 1–1,5 mm. de côté ou plus, et surtout quand on en réunit plusieurs sur une même lame ou dans un même flacon, de nombreux éléments musculaires se changent en macrophages sans intervention expérimentale. Au contraire, s'ils sont petits ($\frac{1}{2}$ ou $\frac{1}{3}$ de mm. de côté) et se trouvent en nombre réduit, la transformation spontanée y devient rare ou nulle.

Mais il est remarquable que divers éléments de ces 'petits' explants se transforment si on les cultive à côté de 'gros' explants. Ceux-ci exercent donc une action à distance sur les premiers. De plus, si on prélève la phase liquide provenant de cultures en flacon Carrel où la transformation est spontanément abondante, et si on l'ajoute à des cultures où la transformation est faible ou nulle, elle provoque une abondante transformation histiocytaire dans ces dernières cultures. Nous avons donc affaire à un facteur actif qui est diffusible et en même temps transmissible par un liquide (1942). La composition de celui-ci est complexe. Constitué à l'origine par du liquide de Tyrode et de l'extrait embryonnaire, il se modifie au cours de la vie des cultures, leur cédant de ses éléments ou, au contraire, s'enrichissant ne divers produits provenant des cellules elles-mêmes, de leurs constituants ou de leur métabolisme.

Une série d'expériences, consistant à traiter et à fractionner le liquide complexe par différentes méthodes chimiques et physiques, ont montré que sa fraction active possède les caractères suivants: elle est thermostable mais est détruite à chaud en milieu fortement alcalin; elle est dialysable et résiste à une dessiccation; enfin, elle est soluble dans l'eau, l'alcool éthylique et l'acétone mais est insoluble dans l'éther de pétrole. La substance active est donc représentée par un ou plusieurs corps de structure simple, à molécule relativement petite et ayant la nature d'un cristalloïde (Chèvremont, 1944, M. et S. Chèvremont, 1945).

Par ailleurs, ce liquide actif, qui correspond à la phase liquide de cultures en flacon où la transformation est spontanée et abondante, s'enrichit en choline pendant la vie de ces cultures et en contient des quantités nettement mesurables. Par la méthode biologique de Brown & Feldberg (1936), Bacq & Chèvremont (1944) ont montré que ce liquide actif renferme en moyenne 25,1γ de chlorure de choline par flacon Carrel, alors que la phase liquide correspondant à un flacon identique mais sans culture n'en contient que 9,9γ et que des éléments musculaires analogues à ceux qui sont explantés, en renferment 1,3γ. Dans un cas où le liquide était faiblement actif, il était nettement plus pauvre en choline (12,6γ). A noter qu'il n'y a pas d'autre ammonium quaternaire du groupe de la choline, du moins en quantité dosable.

Ensuite, on a éliminé aussi électivement que possible du liquide étudié, la choline, afin de voir si celle-ci est responsable du pouvoir transformateur (M. et S. Chèvremont, 1944, 1945). Cette substance a été éliminée par deux méthodes: précipitation à l'acide chloroplatinique ou destruction par une enzyme, la choline-oxydase (préparée à partir du foie de poule ou de rat par la technique de Fr. et M. Bernheim, 1938). Quand on supprime la choline du liquide capable de provoquer la transformation histiocytaire, on le prive de son activité. Par contre, il suffit de le recharger ensuite en choline pour lui faire retrouver tout son pouvoir.

On peut donc conclure de ces expériences que dans la phase liquide baignant des cultures en flacon où la transformation histiocytaire est spontanément abondante, de la choline apparaît en quantité nettement mesurable. Ce corps, qui paraît bien être libéré par les cellules elles-mêmes (voir plus loin), constitue le principe actif du liquide lorsque celui-ci provoque une abondante transformation dans d'autres cultures auquel il est ajouté.

Dans une autre série d'expériences, M. & S. Chèvremont (1945) ont entrepris d'inhiber la transformation histiocytaire qui se produit spontanément dans les cultures (explants de muscles squelettiques ou de tissu conjonctif sous-cutané atteignant un certain volume). L'action de la choline, qui semblait probable, a été mise immédiatement en évidence par l'addition aux cultures de choline-oxydase, qui détruit d'une manière élective la choline préexistante ou apparaissant secondairement. Dans ces conditions, la transformation histiocytaire est inhibée dans une très large mesure et parfois même supprimée complètement. Par exemple, la moyenne approximative du nombre des macrophages nouvellement formés (en cultures de muscles traitées par de la choline-oxydase de foie de poule) tombe de 249 dans les contrôles à 22 dans les cultures traitées par de l'enzyme à $\frac{1}{30}$. Aucun macrophage ne se forme dans les cultures qui ont reçu de l'enzyme à $\frac{1}{18}$. Si on traite par du ferment préalablement inactivé, la transformation spontanée se manifeste normalement. La destruction de la choline inhibe donc la transformation histiocytaire spontanée dans ces cultures.

D'un autre côté, de la choline pure ajoutée expérimentalement à des cultures préparées de telle sorte que la transformation spontanée y soit faible ou nulle ('petits explants'), y détermine de nombreuses cellules à devenir des macrophages, comme nous l'avons vu plus haut.

Ces divers résultats conduisent à la conclusion générale suivante: la choline est le facteur déterminant de la transformation histiocytaire spontanée qui se produit dans les cultures *in vitro* de muscles squelettiques et de tissu conjonctif sous-cutané. Ces faits sont importants non seulement pour les cellules histiocytaires, mais aussi d'un point de vue général. Ils démontrent qu'une substance chimique est capable de changer des éléments cellulaires à caractères nettement spécifiques, tels que les éléments musculaires, en d'autres cellules: les macrophages, qui sont doués de structure et de propriétés caractéristiques mais tout-à-fait différentes de celles des premiers. Très vraisemblablement, c'est le même déterminisme chimique qui intervient dans les cultures de cellules d'autres types, comme les cellules entoblasto-vitellines, les cellules musculaires lisses et d'autres encore. En outre, on peut se demander si les différents moyens qui ont été employés avec plus ou moins de succès dans les cultures en vue d'y provoquer la transformation histiocytaire et qui ont été cités plus haut, n'aboutissent pas finalement à la formation de choline? Il serait intéressant de vérifier cette hypothèse par des recherches biologiques et biochimiques.

Dans le cas des observations de Weiss (1944), faites sur des cultures de fragments de nerfs périphériques ou de ganglions spinaux, la transformation histiocytaire de fibroblastes et de cellules de Schwann appartenant à ces cultures est activement favorisée par la liquidité du milieu et semble être due, selon l'auteur, à un facteur physique: au fait que ces cellules fusiformes entrent en contact avec une surface lisse. Il serait également intéressant de savoir si de la choline est présente, en quelle quantité elle existe, et si elle intervient dans ces derniers processus ou les favorise. D'après Guggenheim (1940), on ignore encore si dans les processus dégénératifs et régénératifs des nerfs, les liquides du corps contiennent plus de choline. On peut aussi se demander si le facteur physique signalé par Weiss comme déterminant, ne correspond pas plutôt à un 'facteur cellulaire' indispensable pour la transformation histiocytaire, comme nous allons le voir ci-après, à un certain état cellulaire nécessaire pour que la choline, éventuellement présente, puisse agir et déterminer une transformation histiocytaire.

VI. INFLUENCE DE FACTEURS CELLULAIRES SUR LA TRANSFORMATION HISTIOCYTAIRE

Si la choline possède une remarquable activité transformatrice, elle n'est pourtant pas capable à elle seule de faire se changer des cellules en macrophages dans tous les cas. L'état de la cellule appelée à se transformer joue un rôle dans la réalisation de ce processus. L'intervention de 'facteurs cellulaires' est nécessaire et, quand ils manquent, la transformation ne se produit pas. C'est le cas pour les cultures de muscles squelettiques qui ont subi une série de repiquages. Les éléments musculaires émigrés de cultures de muscles squelettiques perdent, comme on le sait, au cours de passages successifs *in vitro*, plusieurs de leurs caractères structuraux et fonctionnels (comme les cellules émigrées de cultures de cœur, etc.). Après quatre ou cinq transplantations, ils prennent l'aspect de 'cellules du type fibrocytaire'. Mais, en même temps, leur propriété de se transformer fréquemment et spontanément en macrophages diminue et finalement disparaît tout-à-fait (Chèvremont, 1939, 1942). Fauré-Fremiet & Garrault (1932) avaient déjà fait cette observation pour le mésenchyme. Pourtant, ces 'fibrocytes' des souches peuvent encore exceptionnellement devenir spontanément des macrophages. Par exemple, ce phénomène s'est manifesté une seule fois, au 22° passage, dans une série de 739 cultures en goutte pendante, du 6° au 30° repiquage. Ce phénomène sporadique qui se produit dans des conditions mal précisées, a été signalé pour des souches de fibrocytes dérivés du cœur, par divers chercheurs (Carrel & Ebeling, 1926; Fischer, 1924; Ephrussi & Hughes, 1930; Parker, 1932; Szantroch, 1932; J. A. Thomas, 1934–8, etc.).

Cette transformation peut aussi être provoquée expérimentalement, mais il est difficile de la reproduire avec une grande fréquence. Elle est alors, comme nous allons le voir, nettement moins fréquente que celle qui se produit dans les cultures de muscles squelettiques ou de tissu conjonctif qui n'ont subi aucun repiquage. C'est pourquoi les cellules du type fibrocytaire des souches, utilisées par beaucoup d'auteurs, constituent en fait un matériel peu favorable pour de telles recherches.

Quand on traite des cellules du type fibrocytaire d'origine musculaire et adaptées à la vie *in vitro* par de la choline pure, cette substance si active par ailleurs, reste sans effet et ne détermine la formation d'aucun macrophage (Chèvremont, 1942, chap. 3). Au contraire, le même liquide complexe que celui qui a été signalé antérieurement, est capable de provoquer une certaine transformation histiocytaire de ces 'fibrocytes', actualisant ainsi la potentialité qu'ils ont conservée (Chèvremont, 1943*a*, *c*). Or ce liquide contient de la choline; puisque cette dernière utilisée seule est inactive sur les souches, le liquide doit contenir d'autres éléments nécessaires à la réalisation de la transformation.

Ces éléments ne proviennent pas des constituants des milieux nutritifs mais des cellules cultivées en flacons qui ont fourni le liquide, c'est-à-dire de cellules qui n'ont pas été repiquées. Ce sont les 'facteurs cellulaires' ou 'facteurs locaux' de la transformation histiocytaire. Ils sont en relation avec un certain état structural et fonctionnel des cellules. Celles-ci doivent donc posséder la capacité potentielle de se transformer, mais cette potentialité s'actualise dans certaines conditions, sous l'influence d'un facteur extrinsèque, la choline. Il est probable que ces facteurs cellulaires se retrouvent toujours dans les cellules et fibres musculaires, les cellules conjonctives et autres qui viennent d'être prélevées à l'embryon pour l'explantation, ainsi qu'au niveau des tissus dans l'organisme, en conditions normales ou pathologiques. Ils résulteraient du métabolisme cellulaire normal ou modifié.

C'est donc la choline qui est l'élément déterminant de la transformation histiocytaire, mais son activité remarquable demande pour s'exercer, la présence de 'facteurs cellulaires', dont la nature est encore mal précisée mais qui existent dans la généralité des cas. Ils paraissent être de nature chimique; peut-être, dans certains cas, sont-ils liés à des conditions physiques.

VII. LA TRANSFORMATION HISTIOCYTAIRE *IN VIVO*

Si diverses cellules peuvent se changer en histiocytes et macrophages dans les cultures *in vitro*, elles peuvent le faire également dans l'organisme. Il est en effet bien admis que pendant l'inflammation, des fibrocytes peuvent subir cette transformation. Même, d'après W. von Möllendorff (1931), les histiocytes du tissu conjonctif normal viennent des fibrocytes. Il semble (Chèvremont, 1942) que les fibres musculaires lisses de l'utérus en involution post-partum peuvent donner naissance à de nombreux macrophages, à la suite des modifications du milieu (vascularisation, etc.).

Dans les foyers de contusion, des éléments musculaires et des cellules conjonctives peuvent probablement aussi se transformer en macrophages. Il en est vraisemblablement de même dans les foyers de dégénérescence ou de régénération musculaires où se trouvent à la fois des macrophages et des plasmodiums musculaires plurinucléés qui ressemblent fort à ceux que produisent les muscles striés embryonnaires cultivés *in vitro*, comme l'ont souligné W. et M. R. Lewis (1917).

Diverses recherches anciennes ont déjà mis en évidence l'existence dans ces foyers d'éléments appelés 'sarcolytes' qui jouent un rôle important dans l'élimination des fibres altérées. Bornons-nous à rappeler ici que ce sont des cellules volumineuses, à un ou plusieurs noyaux, qui sont capables d'englober, de phagocyter des fragments musculaires en dégénérescence et qui contiennent parfois aussi des gouttelettes lipidiques. Leur nombre peut être élevé. Si plusieurs auteurs admettaient que les sarcolytes étaient d'origine musculaire, d'autres pensaient qu'ils provenaient de cellules émigrées, à partir surtout des vaisseaux sanguins ou parfois du conjonctif voisin. Par exemple, en ce qui concerne les processus de régression de la queue des têtards lors de la métamorphose, Duesberg (1906) considérait que les sarcolytes correspondaient à des éléments émigrés tandis que Metchnikoff (1892) attribuait à leur noyau une origine musculaire. Cette question de la régénération des muscles striés a fait l'objet, plus récemment, des travaux de Pfuhl (1937), Speidel (1938), Clark (1946), Clark & Wajda (1947), Betz (1947*a*, *b*).

La nature des sarcolytes a été précisée et depuis quelques années, ces éléments sont considérés comme ressemblant à des histiocytes ou comme étant des cellules histiocytaires. Mais de nombreux auteurs n'imaginaient pas, ou n'osaient pas admettre, que des éléments aussi hautement différenciés et spécialisés que les fibres musculaires striées puissent donner naissance à des histiocytes et macrophages. C'est ainsi que, entre autres, Pfuhl écrivait en 1937, au cours d'une étude sur les fibres musculaires striées (pp. 573–574): 'Mit unseren modernen Anschauungen über Differenzierung und Entdifferenzierung von Zellen ist es nicht in Einklang zu bringen, dass aus einem so hochdifferenzierten Material, wie es die quergestreifte Muskelfaser darstellt, phagocytäre Zellen entstehen, die als "Sarkolyten" den degenerierten Sarkolemminhalt auffressen.' De son côté Clark écrit (1946, p. 33): 'The conception that degenerating muscle fibres may give rise to histiocytic elements is difficult to accept in view of the highly specialized nature of striated muscle', mais il ajoute aussitôt: 'Nevertheless, it receives some support from recent work by Chèvremont (1940) who claims to have observed the direct transformation *in vitro* of myoblastic cells into typical histiocytes.'

En effet, à la lumière des faits que j'ai établis par la culture de muscles *in vitro* et qui ont été cités plus haut, on peut penser que ces sarcolytes correspondent à des macrophages ou à des cellules géantes de nature histiocytaire dont le plus grand nombre proviennent d'une transformation histiocytaire de fibres musculaires lésées, comme je l'ai déjà signalé antérieurement. Naturellement, certains des sarcolytes peuvent dériver aussi de cellules histiocytaires préexistantes dans le conjonctif, de monocytes sortant des vaisseaux, de divisions d'histiocytes ou encore éventuellement de la transformation histiocytaire de fibrocytes.

Des exemples d'une telle transformation histiocytaire frappant des éléments de nature musculaire certaine ont été trouvés *in vivo* par Firket et ses collaborateurs, dont les intéressantes observations confirment ainsi les résultats que j'ai obtenus *in vitro*. Firket & Cornil (1944) ont en effet démontré que dans un cas de granulomatose musculaire humaine, des fibres musculaires striées de la paroi abdominale se sont transformées en histiocytes et macrophages. Firket & Brabant (1946) ont ensuite décrit, chez l'Homme, un cas de réticulo-granulomatose maligne (réticulo-endothéliose) d'origine musculaire. Cette affection frappait les tissus histiocytaires des moelles osseuses et de la rate, mais surtout la langue. Au niveau de cette dernière, les auteurs ont observé un aspect peu commun de myosite, sans extravasation cellulaire mais avec d'abondantes cellules géantes plurinucléées. Ces cellules géantes, qui sont de nature histiocytaire, proviennent avec certitude des bouts libres de fibres musculaires en dégénérescence.

Tout récemment, Betz (1947*a*, *b*) a étudié la dégénérescence et la régénération *in vivo* des fibres musculaires striées après ischémie, et il a précisé l'origine des cellules histiocytaires intervenant au cours de ces processus. Il a montré notamment que beaucoup de sarcolytes des anciens auteurs, qui sont en réalité de nature histiocytaire, sont d'origine musculaire, comme le prouve entre autres leur capacité marquée d'athrocyter des colorants vitaux.

Dans les processus dégénératifs et régénératifs des nerfs, il est possible que la présence de nombreux macrophages soit due également à des phénomènes de transformation histiocytaire (fibrocytes du tissu conjonctif; cellules de Schwann). Certains faits plaident en faveur de cet opinion, mais Weiss écrit en 1944 (p. 219): 'In spite of extensive histological studies of nerve regeneration in the body, I have been unable thus far to ascertain whether or not transformation of mobilized sheath cells (*Schwann cells*) into macrophages in injured nerves may occur.'

Enfin, dans de nombreuses réactions inflammatoires localisées ou généralisées, on doit admettre actuellement que des cellules histiocytaires peuvent se former sur place, par exemple à partir de fibrocytes, indépendamment de celles qui se trouvaient préalablement au niveau du foyer inflammatoire ou qui y ont émigré.

VIII. ORIGINE DE LA CHOLINE ET POSSIBILITÉS DE SON INTERVENTION *IN VIVO*

D'où vient cette choline qui est capable de provoquer la transformation histiocytaire? Dans les cultures de muscles squelettiques, il est probable qu'elle provient de l'hydrolyse des lécithines musculaires. Les muscles contiennent en effet de 3 à 6% de lécithines (chez le Bœuf, d'après P. Thomas (1936). Cette hydrolyse se produirait dès le début de la vie *in vitro*, spécialement dans les explants atteignant des dimensions suffisantes. Ce phénomène pourrait être en relation avec l'action d'enzymes.

Beaucoup de tissus et d'organes renferment *in vivo* des quantités mesurables de choline. Mais, chose importante, on a montré que de la choline peut se former secondairement en quantités bien mesurables ou augmenter considérablement dans certaines circonstances. Par exemple, du foie simplement abandonné pendant 5 heures en contient 136 à 164γ chez le Chien et 213 à 773γ chez le Bœuf, tandis que l'organe frais en est privé ou n'en contient au plus que de très faibles quantités mesurables (Strack, Neubaur & Geiszendörfer, cités par Guggenheim, 1940). Il en est de même pour le muscle. Il importe de souligner que ces concentrations de choline qui peuvent se rencontrer au niveau des tissus sont du même ordre de grandeur que celles qui sont actives *in vitro*. Exprimées en γ par cc., ces dernières s'élèvent à 123–412γ de chlorure de choline, dans la grande majorité des expériences faites sur les cultures par M. Chèvremont en 1943.

Dans les tissus, la choline peut avoir une origine diverse. Elle peut provenir de la décomposition de l'acétylcholine libérée par les terminaisons nerveuses parasympathiques. Elle peut aussi se former, sous l'effet d'enzymes, à partir de lécithines présentes dans les cellules à l'état d'enclaves figurées ou combinées à d'autres constituants protoplasmiques. Sphyngomyélines, carnitine, acides oxyaminés participant à la constitution des protéines et même des produits de décomposition semblent aussi pouvoir lui donner naissance (voir Guggenheim, 1934, 1940; P. Thomas, 1936, 1946; Cristol, 1942, etc.); il en serait de même pour certains précurseurs encore peu connus, comme le pensent Kahane & Lévy (1939, etc.). Remarquons aussi que la rate, riche en cellules histiocytaires (tissu réticulé, endothélium sinusoïde veineux, cellules libres) se caractérise aussi par sa richesse particulière en choline (Kahane & Lévy, 1939, etc.).

Différents arguments, parfois indirects, sont aussi en faveur de l'intervention de la choline dans la transformation histiocytaire *in vivo*. Ils peuvent être tirés d'observations souvent réalisées dans d'autres domaines et avec d'autres buts que ceux dont il est question ici.

A la suite de travaux montrant que les lécithines, lipides phosphorés, confèrent à l'animal une plus grande résistance à l'infection tuberculeuse, Fiessinger (1923) entreprit d'étudier le rôle de lipases dans cette résistance. Par l'injection de lécithines ou de cire, il obtint les résultats suivants. Des cobayes qui ont reçu des injections répétées de lécithine, en solution dans l'huile d'amandes douces, montrent une augmentation considérable des ganglions lymphatiques iliaques, mésentériques et médiastinaux. Ils sont infiltrés de graisse, mais sans inflammation. La rate est notablement accrue en dimensions par rapport aux animaux témoins. Pour les autres organes, rien n'a été observé, si ce n'est une augmentation considérable des dimensions du pancréas et une infiltration graisseuse des régions para- et prévertébrales. Par contre, chez des cobayes traités dans les mêmes conditions par de la cire en solution dans l'huile d'amandes douces, rien de spécial n'a été constaté. Ganglions lymphatiques et rate avaient un aspect normal.

Des observations de Pirolli (1942) peuvent aussi être citées. Cet auteur a montré que des 'lipoïdes acétono-insolubles', extraits de muscle, de rate ou de testicule, exercent une influence stimulante sur le système réticulo-endothélial du lapin. Ces lipoïdes ne causent aucun changement dans les autres organes, sauf une dégénérescence du sarcolemme. Ensuite, Pirolli a signalé que, chez les animaux injectés de lipoïdes acétono-insolubles extraits du foie, il se produit une augmentation considérable du nombre des cellules réticulo-endothéliales de la rate. Par l'injection d'extraits de rein, la rate et ses trabécules s'épaississent. Les deux sortes d'extraits provoquent une dégénérescence du sarcolemme. Il faut remarquer, d'un côté, que les lipoïdes acétono-insolubles de ces extraits doivent certainement être constitués, pour une large part, par des lécithines et que, d'un autre côté, la cire employée par Fiessinger dans quelques-unes de ses expériences, ne contient pas de choline dans ses molécules, à l'opposé des lécithines.

A la lumière des faits nouveaux signalés plus haut, on peut proposer l'interprétation suivante des expériences de Fiessinger et de Pirolli. Les lécithines injectées chez l'animal seraient hydrolysées, partiellement ou non, et libéreraient de la choline. Cette choline provoquerait la formation de nouvelles cellules histiocytaires. Il serait intéressant de connaître davantage les phénomènes cellulaires qui se produisent dans ces cas, leur électivité et leur mécanisme.

Étudiant les relations entre les 'lipoid storage diseases' et les anémies hémolytiques, Tompkins (1943) a réalisé des injections intraveineuses répétées de lécithine à des lapins. Il a observé une infiltration généralisée de macrophages, une augmentation des globules blancs, une baisse des globules rouges ainsi qu'une hyperplasie de la moelle osseuse et de la splénomégalie.

Une observation de Firket & Cornil (1944), qui vient déjà d'être citée, constitue un argument direct en faveur de l'intervention de la choline dans la production de la transformation histiocytaire d'éléments musculaires *in vivo*. En effet, des dosages biologiques ont permis de déceler la présence d'une quantité élevée de choline dans la région musculaire pathologique. Dans cette dernière, on a trouvé une valeur de 38γ de chlorure de choline par gramme de tissu, alors que les muscles normaux voisins n'en contiennent que 1γ. Le premier chiffre est à rapprocher de la teneur en choline de la phase liquide qui recouvre les cultures de muscles squelettiques en flacon où la transformation histiocytaire est spontanée et abondante. Ce liquide, qui est lui-même actif *in vitro*, contient 31·5γ de choline par c.c.

Il y aurait lieu de poursuivre des recherches dans cette voie, au point de vue normal comme au point de vue pathologique, et de voir si la choline, ou éventuellement d'autres substances, interviennent dans d'autres circonstances.

IX. DÉTERMINISME DE LA PRODUCTION DES CELLULES ÉPITHÉLIOÏDES ET GÉANTES

Comme nous l'avons rappelé, les cellules histiocytaires peuvent prendre, dans certains cas, l'aspect de cellules épithélioïdes et de cellules géantes. On peut observer ces cellules particulières dans les cultures *in vitro*. On les rencontre aussi dans l'organisme, autour de substances ou de corps étrangers non résorbables ou résorbables lentement, qui ont été introduits accidentellement ou expérimentalement (fils de coton, etc.), ou encore qui s'accumulent en certains points à la suite d'un trouble métabolique (dépôts d'urate, de cholestérol, etc.). Elles existent également dans les inflammations chroniques, tuberculose, syphilis, mycoses, lèpre, etc. Leur rôle est important.

Quant à leur origine et à leur nature, elles ont été longtemps discutées. Mais les travaux de Maximow (1927), de M. R. et W. Lewis (1917), de Sabin (1927–38), réalisés surtout en culture de tissus ou par la méthode des colorations supravitales, ont établi que les cellules épithélioïdes dérivent principalement des monocytes, lymphocytes, 'clasmatocytes', et en général de cellules réticulo-endothéliales, occasionnellement aussi de fibrocytes. De leur côté, les cellules géantes proviennent des cellules épithélioïdes, soit par fusion de cellules séparées, soit à partir d'une cellule unique où se produisent des divisions amitotiques du noyau, sans séparation du cytoplasme (Barta, 1926; W. Lewis, 1928; Levi, 1934, etc.). Les cellules épithélioïdes sont donc des cellules histiocytaires, des macrophages qui ont pris un type particulier; les cellules géantes sont également de nature histiocytaire.

Quel est le déterminisme de cette réaction cellulaire? Dans les cultures de tissus, la formation de cellules géantes aux dépens de cellules histiocytaires est favorisée par la sous-oxygénation locale, en milieu pauvre en extrait embryonnaire (Barta, 1925, 1926; W. Lewis, 1928).*

C'est particulièrement dans le cas de la tuberculose que le déterminisme de ces réactions cellulaires si caractéristiques a été étudié. Il semble bien que ce phénomène soit sous la dépendance de substances apportées par les bacilles de Koch. Ce sont tout d'abord les divers lipides que le bacille tuberculeux contient en abondance et spécialement les lipides renfermant un acide gras particulier, l'acide phtioïque, qui ont paru représenter les substances actives (Sabin et ses collaborateurs; Roulet, 1939).† Cette conception est très discutée surtout depuis les observations de Boissevain et les dernières publications de Sabin et ses collaborateurs (1938, etc.). Actuellement, on pense bien que ce sont des matières protéidiques insolubles spéciales du bacille tuberculeux qui sont essentiellement les facteurs déterminants (Boivin, Delaunay, Lasfargues, Pagès & Vendrely, 1946; Boivin & Delaunay, 1947).

Après une réaction passagère à polynucléaires dont l'appel est provoqué par les matières glucidiques microbiennes (Delaunay), il y aurait ainsi une sorte de réaction cellulaire en deux temps, réglée par un déterminisme chimique. On peut concevoir que le premier temps serait marqué par l'émigration de cellules histiocytaires et la formation de nouvelles cellules de ce type sous l'influence de la choline, celle-ci étant libérée à partir d'éléments tissulaires, à l'endroit même de l'infection. Par le second processus, des éléments chimiques apportés par les bacilles imposeraient aux cellules histiocytaires le type particulier à cette infection, peut-être avec l'action favorisante de la sous-oxygénation locale (Chèvremont).

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* Selon Törö (1947), les cellules histiocytaires de différents types (histiocytes, polyblastes, cellules adventitielles, etc.) se transforment l'une dans l'autre sous l'influence de l'histamine.

† L'étude des lipides divers du bacille de Koch a été faite surtout par Anderson (1932) et par Macheboeuf (1935). C'est notamment le premier qui a découvert l'acide phtioïque.

X. L'ÉTAT HISTIOCYTAIRE

La nature des cellules histiocytaires a déjà fait l'objet de nombreuses discussions. Dans la conception classique, elles sont souvent considérées comme des cellules non différenciées, restées ou retournées à l'état embryonnaire. Cette conception est inexacte, notamment parce que ces cellules possèdent des caractères structuraux et biologiques très particuliers et bien individualisés, comme nous l'avons vu. D'autre part, elles n'appartiennent pas à une souche spécifique, génétiquement définie.

Les recherches récentes de cytologie expérimentale ont amené à modifier la conception classique sur la nature de ces cellules. Etendant et précisant une conception ébauchée par d'autres auteurs (Levi, W. von Möllendorff, Ephrussi, Thomas), j'ai montré (Chèvremont, 1939–45) que les cellules histiocytaires constituent, en réalité, un état cellulaire particulier, caractérisé surtout par son aspect fonctionnel, que beaucoup de cellules différenciées et de types divers peuvent prendre: c'est l'état histiocytaire. Certaines cellules possèdent cet état dès leur origine (cellules du tissu réticulé et de l'endothélium sinusoïde, par exemple). D'autres, au contraire, s'y trouvent par acquisition secondaire, à la suite du passage à l'état histiocytaire de cellules diverses, telles que fibrocytes, éléments musculaires, cellules épithéliales, etc.

En prenant l'état histiocytaire, des cellules acquièrent des propriétés nouvelles et importantes, très utiles pour l'organisme. Elles sont notamment plus résistantes et mieux équipées, et ont ainsi plus de chance d''échapper à la mort'. Cette notion du passage à l'état histiocytaire présente un intérêt considérable aux points de vue histophysiologique et histopathologique.

XI. CRITIQUE DU TERME "SYSTÈME RÉTICULO-ENDOTHÉLIAL"

Les cellules dont il est question ici sont souvent appelées réticulo-endothéliales et le vaste ensemble qu'elles constituent, le système réticulo-endothélial ou S.R.E. Ce terme de 'réticulo-endothélial' doit être abandonné parce que, pour plusieurs raisons, il est erroné et prête à confusion.

En effet, les cellules endothéliales des vaisseaux sanguins communs n'appartiennent nullement à la catégorie des cellules histiocytaires et ne donnent pas naissance à des histiocytes, comme le soutient notamment Sabin (1921) contrairement à Maximow (1924), Lewis (1928), Levi (1934). Au cours d'une étude systématique de différents tissus, j'ai vérifié que, dans les conditions habituelles, les cellules de l'endothélium vasculaire commun ne se colorent pas par le Rouge neutre (technique postvitale et en culture) et ne se transforment pas en macrophages, *in vitro* (Chèvremont, 1942). Seul l'endothélium particulier des sinus veineux est de nature histiocytaire, et peut-être, exceptionnellement, l'endothélium des veines pulpaires de la rate (Petersen, 1935).

En outre, différents auteurs ont confondu le réticulum des cellules histiocytaires avec le réticulum des fibres réticulées et grillagées. Ces deux réseaux peuvent coexister mais sont d'une nature tout-à-fait distincte et sont, dans certains cas, indépendants l'un de l'autre. C'est en faisant cette confusion que Volterra (1925) et d'autres finissaient par faire rentrer dans le S.R.E. des éléments qui n'avaient rien à y voir, tels que la membrane basale des épithéliums, etc. Enfin, il existe des histiocytes qui ne sont ni réticulaires ni endothéliaux: ceux du conjonctif sous-cutané par exemple.

Il vaut donc mieux employer l'expression: le *système histiocytaire* (Petersen, 1935), suivant l'ancienne dénomination d'Aschoff (1924), mais dans un sens plus large, ou encore système des cellules du type histiocytaire (Levi, 1935). De même, il est préférable d'appeler du terme général de cellules histiocytaires toutes les cellules appartenant à ce système.*

XII. CONCEPTION GÉNÉRALE DU FONCTIONNEMENT DU SYSTÈME HISTIOCYTAIRE

L'ensemble des faits observés *in vitro* et *in vivo* et des arguments qu'on peut en tirer, permettent de proposer la conception générale suivante (Chèvremont, 1945). Se mobilisant, les cellules histiocytaires peuvent se rendre pratiquement en n'importe quel point de l'organisme. Elles peuvent aussi se multiplier par mitose, à l'opposé des leucocytes qui ne se divisent plus guère. D'autres peuvent être apportées par le sang (monocytes). Elles peuvent donc se concentrer, vraisemblablement attirées en certains points par chimiotactisme. De plus, phénomène important, elles peuvent se former sur place *de novo* et en grand nombre, aux dépens de différentes cellules.

La transformation histiocytaire et vraisemblablement la formation des cellules histiocytaires en général sont réglées par un déterminisme chimique. C'est la choline qui en est le facteur déterminant. Des cellules diverses sont amenées à se changer en histiocytes et macrophages, par l'action de cette substance chimique, acquérant ainsi de nouveaux caractères structuraux et fonctionnels. La choline serait libérée au niveau même des tissus, dans certaines circonstances normales ou pathologiques. Sous son influence, quand la concentration locale est suffisante, des histiocytes et macrophages se forment *in situ*, en quelques heures, aux dépens de fibrocytes, de cellules et éléments musculaires, probablement d'autres encore et peut-être de cellules épithéliales. Elles acquièrent ainsi des potentialités multiples si importantes pour la défense de l'organisme. Des facteurs cellulaires joueraient un certain rôle dans la réalisation de ce processus chimique.

Cette conception est plus générale et rend mieux compte de l'ensemble des faits que l'hypothèse d'un fonctionnement neurochimique du système histiocytaire, suggérée par Thomas (1935–8) en se basant sur ses résultats obtenus avec les cellules entoblasto-vitellines. S'il est vrai que l'acétylcholine ou de la choline peuvent résulter de l'activité de nerfs cholinergiques et pourraient intervenir dans le fonctionnement du système histiocytaire, ce n'est là qu'une partie de la réalité, puisque de la choline peut se former en quantité appréciable à peu près dans tous les tissus, dans certaines circonstances pathologiques ou non, indépendamment de tout élément nerveux.

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* Précisons à ce propos une question de nomenclature. Nous avons défini plus haut histiocytes et macrophages. Les histiocytes épars dans le tissu conjonctif sont les histiocytes proprement dits, terme qui nous paraît préférable à celui de clasmatocytes, employé par Ranvier. Quant à la cellule réticulée par exemple, on peut dire que c'est un histiocyte fixe mais susceptible de se mobiliser, de devenir un macrophage. Enfin, le monocyte est un histiocyte qui, se trouvant en milieu liquide, le sang, est arrondi. Tout récemment, Bessis (1947) vient de discuter cette question du point de vue hématologique, en étudiant la cellule réticulaire normale et pathologique.

Secondairement, des cellules histiocytaires, des macrophages, peuvent prendre, dans certaines conditions locales, un type particulier, celui de cellules épithélioïdes et de cellules géantes, peut-être sous l'influence de substances chimiques.

XIII. RÉSUMÉ

Après avoir rappelé brièvement la signification et l'importance des cellules réticulo-endothéliales ou histiocytaires, qui sont très répandues et qui, grâce à leurs potentialités multiples, jouent un rôle considérable dans l'organisme, à la fois dans les conditions normales et pathologiques, le présent travail résume et situe les principales connaissances qui ont été acquises récemment dans le domaine de la biologie de ces cellules.

Tout d'abord, plusieurs *caractères cytologiques et propriétés biologiques des histiocytes et macrophages* ont pu être précisés ou établis (étude de leur structure et de leurs fonctions en relation avec l'origine et la localisation; histochimie de leurs lipides; athrocytose et phagocytose; absorption et stockage de vitamines, etc.).

Un nouveau type d'histiocyte a été individualisé et décrit: les *histiocytes graisseux*, d'où dérivent les cellules adipeuses de la graisse de formation secondaire.

Les cellules histiocytaires présentent aussi la propriété importante de pouvoir se former *de novo*, à partir de cellules d'un tout autre type; c'est la *transformation histiocytaire*. Celle-ci se produit *in vitro*, aux dépens de lymphocytes, de cellules du type fibrocytaire des souches de cœur ou de tissu conjonctif, de mésenchyme, d'entoblaste vitellin, de fibrocytes du tissu sous-cutané à différents âges, d'éléments des muscles squelettiques ou lisses, de cellules de Schwann. Cette transformation spontanée est particulièrement fréquente dans les cultures non repiquées de muscles squelettiques ou de conjonctif.

On peut *provoquer expérimentalement* la transformation histiocytaire *in vitro* par des moyens variés et avec des résultats divers. Des substances chimiques pures se sont révélées actives; ce sont l'arsenic (sur les fibrocytes de souches), le sulfate d'atropine (sur les cultures de cœur ou de mésenchyme) et surtout des ammoniums quaternaires du groupe de la choline (sur des cultures d'entoblaste vitellin de la vésicule ombilicale) et la choline et l'acétylcholine (sur des cultures non repiquées de muscles squelettiques ou de conjonctif sous-cutané).

L'important problème du *déterminisme de la transformation histiocytaire spontanée* a été étudié. Dans le cas des cultures de tissu conjonctif sous-cutané et dans celui des cultures de muscles squelettiques, c'est la *choline* qui est le facteur déterminant de la transformation *in vitro* des éléments conjonctifs ou musculaires en macrophages.

Des '*facteurs cellulaires*', encore peu connus, jouent un rôle dans ces processus; l'état de la cellule appelée à se transformer intervient.

La transformation histiocytaire peut aussi se produire *in vivo* au cours de divers phénomènes (nombreuses réactions inflammatoires localisées ou généralisées; dégénérescence et régénération des muscles; certaines tumeurs musculaires, etc.). Il est possible que ce

soit également la choline qui intervienne *in vivo*. Plusieurs arguments en faveur de cette thèse sont cités, mais les recherches doivent être poursuivies pour tenter de déceler les substances éventuellement en cause dans divers cas.

De la choline peut apparaître et se former secondairement au niveau des tissus, dans différentes circonstances, *in vivo* et *in vitro*.

L'aspect de *cellules épithélioïdes et de cellules géantes* que prennent dans certains cas des cellules histiocytaires, est probablement déterminé par des substances chimiques, peut-être avec l'influence favorisante de la sous-oxygénation locale. Dans la tuberculose, il semble bien que leur formation soit due à des substances protéidiques insolubles provenant des bacilles de Koch.

La notion nouvelle de l'*état histiocytaire* est exposée. C'est l'état cellulaire particulier que beaucoup de cellules différenciées et de types divers prennent en se changeant en histiocytes et macrophages. Il est caractérisé surtout par son aspect fonctionnel.

Parce que, pour différentes raisons, il est erroné et prête à confusion, le terme 'réticulo-endothélial' doit être abandonné. Il vaut mieux dire: *système histiocytaire*, cellules histiocytaires.

Enfin, une *conception générale* nouvelle du fonctionnement du système histiocytaire est proposée.

XIV. BIBLIOGRAPHIE*

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THE DEVELOPMENT AND FATE OF THE CORPUS LUTEUM IN THE VERTEBRATE SERIES

By R. J. HARRISON
Anatomy Department, Charing Cross Hospital Medical School, London

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I. INTRODUCTION

The corpus luteum is the term applied to the endocrine gland which develops in the ruptured ovarian follicle after maturation and discharge of the ovum. The corpus luteum is so called because of the yellow colour which the gland exhibits in its fresh state in some mammals, due to the presence of a carotenoid pigment in the luteal cells. The corpus luteum produces the hormone progesterone, which influences secondary sex organs and is concerned with the progestational changes in the uterus. Its discovery is attributed by Solomons & Gatenby (1924) to Volcherus Coiter in 1573, but there is evidence that Vesalius had observed a corpus luteum in the ovary of a young girl some thirty years previously. The first good description of the gland was given by Regner de Graaf in *De Mulierum Organis Generationi Inservientibus*, published in 1672 (translated by G. W. Corner, 1943*b*).

Corpora lutea normally develop from all ruptured follicles, but if fertilization of the ovum and subsequent implantation in the uterus of the blastocyst do not occur, the corpora lutea soon degenerate. Such corpora lutea are referred to as corpora lutea of the cycle, or corpora lutea of ovulation. However, if implantation of the blastocyst occurs, the corpora lutea persist longer and become corpora lutea of pregnancy. In some mammals the corpora lutea of ovulation are short-lived, and are probably non-functional. However, in these forms the corpora lutea may be activated by coitus with a sterile buck, by mechanical stimulation of the cervix, or by a copulation which does not result in pregnancy. Such corpora lutea are called corpora lutea of pseudo-pregnancy.

There have been numerous reviews on the development of the corpus luteum during the last fifty years. Those by Sobotta (1895, 1896, 1899, 1902), Marshall (1905), Corner (1919) and Pratt (1935) are primarily concerned with the now century-old controversy about the origin of the luteal cells. The reviews by Marshall (1922), Asdell (1928), Hett (1933) deal with the morphology of the corpus luteum, and valuable information may be obtained from the more recent works by Allen, Danforth & Doisy (1939), Asdell (1946) and Courrier (1947).

It is not proposed to reiterate the views of the early protagonists in the controversy concerning the origin of the luteal cells, for they are only of historical interest. This article is intended to be a brief statement on the histological and cytological changes that have been described as occurring during the development of the corpus luteum. For the most part observations concerned with the physiological, biochemical or endocrinological aspect of the activity of the corpus luteum have been omitted. It is hoped that this survey will enable the ever-increasing number of workers on reproductive anatomy and physiology to ascertain the relative value of published descriptions of the development of the corpus luteum in any particular species. Papers are only described in some detail if the matter presented fulfils the criteria stressed many years ago by Corner (1919), which are so necessary for any description of the changes in the corpus luteum to be of value. It is essential for any series of specimens to be individually timed from the date of ovulation, to be from a normal ovary, and to be related to the state of affairs in the reproductive tract or to the degree of development of the zygote, trophoblast or placenta.

II. THE CORPUS LUTEUM IN VERTEBRATES OTHER THAN MAMMALS

The reader should consult Asdell (1928) for a previous review of the literature concerned with the luteal-like structures found in the ovaries of lower vertebrates. Descriptions of luteal-like bodies developing in the post-ovulatory follicle of the fowl are given by Pearl & Boring (1918), Hett (1923), Yocom (1924), and Davis (1942). Pearl & Boring state that the 'corpus luteum' of the hen develops from the theca interna cells. Davis maintains that the post-ovulatory follicle in birds is not identical with the corpus luteum of mammals because there is no hypertrophy or proliferation of the granulosa cells. The post-ovulatory follicle quickly shrinks to half its original size and contains a mass of greatly vacuolated granulosa cells and phagocytes. The thecal cells also become vacuolated and the thecal layer frequently contains hyalin.

Among the reptiles it has been shown that the follicular epithelium remains in the spent follicle to form a corpus luteum in *Seps chalcides* (Mingazzini, 1893), *Anguis fragilis* (Lucien, 1903), *Lacerta agilis* (Hett, 1924), *L. viridis* (Boyd, 1940–1), *L. vivipara* and other viviparous lizards (Weekes, 1934), *Zootica vivipara* (Cunningham & Smart, 1934) and *Hoplodactylus maculatus* (Boyd, 1940–1).

In *Hoplodactylus* and *Lacerta vivipara* the fibroblasts penetrate between the individual luteal cells and also form septa, but in the former no blood vessels are found in the corpus luteum. No penetration of the corpus luteum by blood vessels occurs in a number of other viviparous lizards (Weekes, 1934). In *Hoplodactylus* there is no overgrowth by thecal tissue of the rupture point of the follicle as occurs in most reptiles. Vacuoles containing lipid develop in the luteal cells of *Hoplodactylus* when the related egg contains an embryo with thirty somites, and the vacuolation steadily increases in amount thereafter. The theca interna plays no part in the development of the corpus luteum in this form. The behaviour of the theca interna apparently varies with the genus of lizard (see Weekes, 1934, for detailed description in certain Australian lizards).

Cunningham & Smart (1934) state that the essential character of the corpus luteum in lower vertebrates is the persistence and hypertrophy of the ruptured follicle. In their opinion the latter only occurs in viviparous forms of lower vertebrates, whereas in the oviparous forms the ruptured follicle shows degenerative changes (see Weekes, 1934). The corpus luteum of reptiles, however, arises independently of the presence or absence of placentation. Since corpora lutea are formed in oviparous reptiles they may have appeared before placentation, and later have acquired a function connected with placentation (Boyd, 1940–1). In reptiles the development of a corpus luteum is not necessarily associated with viviparity only, but also with the mere retention of eggs in the oviduct (Rahn, 1938). Luteal tissue found in the ovaries of the viviparous snakes *Thamnophis* and *Potamophis* is apparently solely derived from the granulosa cells, the theca interna cells providing the supporting tissue. Such luteal tissue is found in the ovaries of seven species of viviparous snakes, two species of oviparous snakes, the horned toad lizard and the snapping turtle. These bodies are well vascularized and are maintained throughout gestation. They degenerate slowly after parturition. In the viviparous snakes *Thamnophis* and *Natrix* the morphological appearance of the corpora lutea antedates their possible utilization as endocrine organs (Bragdon, 1946). It will be seen from these reports that further investigation of the post-ovulatory follicles in lower vertebrates is required before a definite statement can be made as to the function of such bodies.

III. THE CORPUS LUTEUM IN THE PROTOTHERIA AND METATHERIA

(1) *Monotremata*

Hill & Gatenby (1926) were the first to investigate the development and fate of the gland in a series of specimens in which the stage of the developing egg was known. They found that the corpus luteum in the Monotremata attained its full growth and activity during the uterine period of development of the egg, and that signs of regression can be found as soon as that phase is completed. They describe three main phases in the developmental history of the gland.

The first phase extends from just before ovulation to the time when cleavage is completed. During this time the corpus luteum becomes solid throughout and herniates through the ovulation point. The original follicular lining becomes folded after collapse of the follicle, with projections of theca externa cells filling the cores of the folds. The membrana propria disappears and the theca interna cells invade the lutealizing granulosa cells. Groups of theca interna cells, presenting a syncytial arrangement, are also to be found about the periphery of the gland. At first the cells have a slightly vacuolated cytoplasm, occasionally with one large vacuole, but towards the end of the first phase the vacuolation becomes very marked. The luteal cells hypertrophy to twice the size of the original follicular cells; their cytoplasm is granular and exceptionally contains a clear vacuolar space, and towards the end of the first phase their nuclei become vesiculated. Capillary invasion commences early and localized extravasations of blood are found in the theca externa. There is no evidence of extensive intra-follicular haemorrhage (Garde, 1930). Mitoses are not found in the granulosa cells at this stage, but a few are present in the theca interna. At the end of the phase a connective tissue reticulum extends throughout the corpus luteum. Hill & Gatenby (1926) believe it to be formed from thecal fibroblasts which migrated inwards from the theca externa. The first phase is therefore characterized by a period of remarkable multiplication and presumed glandular activity on the part of the theca interna cells. Solomons & Gatenby (1924) state that the theca interna cells increase mitotically before and after ovulation, but do not hypertrophy like the granulosa cells, and take no part in luteal cell formation. They do not form true connective tissue, neither do they become spindle-shaped, and they never contain visible rods and granules. These authors believe that the theca externa cells give rise to the connective tissue of the corpus luteum.

The second phase, during which the luteal cells reach their height of cytological development, extends from the completion of cleavage up to the time of the formation of the blastocyst. Occasionally the cytoplasm of the luteal cells contains a clear spherical vacuole, but such vacuoles are apparently not to be compared with the fat-containing vacuoles of the eutherian luteal cells. Some cells show spherical spaces in the cytoplasm containing a deeply staining homogeneous coagulum. A feature of the fully differentiated luteal cell is the presence of numerous large mitochondria which sweep round the cell, lying between the juxta-nuclear cytoplasm and the periphery. The luteal cells shrink slightly towards the end of the phase, and many become elongated with their extremities presenting a broken-up flocculent appearance. The theca interna cells are found in the same situations, but their nuclei are smaller and vacuolation is less marked. The whole gland presents a more open appearance during this phase, due mainly to the larger size of the capillaries.

The third phase covers a final period of retrogression. Even in the early blastocyst stage numbers of degenerating, heavily staining luteal cells are present; later they increase in number. Colloidal and granular degeneration of the luteal cells is well advanced by the time the egg is laid. Cytoplasmic vacuolation of the luteal cells now becomes less obvious. Leucocytes are common in the luteal and connective tissues. Spheres of a granular structure can be seen isolated by some peculiar process of cell disintegration in the vacuolar spaces. The theca interna cells are less obvious during this final phase, their cytoplasm is shrunken and the cells are obviously inactive. Although the connective tissue increases during this period there is no evidence that the theca interna cells take on a fibroblastic activity and give rise to any part of the connective tissue. Hill & Gatenby state that no liquid or true fat is formed in the luteal cells, which is in contradistinction to the state of affairs in the eutherian corpus luteum.

A corpus luteum from *Echidna* is described by Hill & Gatenby (1926) which possesses a large central cavity. The state of the uterine glands indicates that this corpus luteum is of an equivalent stage to that described under the first phase for *Platypus*.

(2) *Marsupalia*

Sandes (1903) was the first investigator to describe the corpus luteum in the Marsupalia. He found that in the native cat, *Dasyurus viverrinus*, the luteal cells become fully developed when the blastocysts are 6·5–7 mm. in diameter and are present in the uteri. There is little intra-follicular haemorrhage and the corpus luteum is fully formed three days after ovulation. The cytoplasm of the luteal cells becomes granular before the third day, and many luteal cells have two or three nuclei, but no mitotic figures are seen. Sandes finds it difficult to distinguish between the parts played by the theca interna and externa. The theca interna is not well developed and forms only the vascular connective tissue support of the gland in his opinion.

At the stage of full development of the luteal cells, on the fifth day, some degree of vacuolation can be seen in the peripheral part of the cytoplasm of the luteal cells. The gland remains in its fully developed state during the greater part of the time that the animal is lactating (7–8 weeks), and thereafter steadily retrogresses. The chief factors in the retrogressive changes are a fatty degeneration of the luteal cells, their removal by leucocytes, atrophy of the blood vessels, and an increase in the amount of connective tissue to form a corpus fibrosum. By the time the young are 10 cm. long, 4 months after birth, there remains no trace of the corpus luteum in the ovary.

The appearances of the corpora lutea of other Australian marsupials have been briefly described by O'Donoghue (1916). Definite evidence of mitotic division of the granulosa cells is claimed during the early changes of lutealization in the bandicoots *Parameles obesula* and *P. nasuta*, and in the American opossum *Didelphys aurita*. The corpus luteum in the koala *Phascolarctos cinereus* remains hollow even when fully grown, and the central cavity does not get filled in until some time after the birth of the young. This condition is apparently limited to this single species in the Marsupalia.

More recently the corpus luteum of the opossum, *Didelphys virginiana*, has been described by Martinez-Esteve (1942). The ovum of the opossum reaches the uterus more rapidly than in any other mammal, and at this time (24 hr.) the corpus luteum has a solid border, but still has a central cavity. The gland is solid by the time of the third and fourth cleavages. Occasional mitotic figures are observed in the luteal cells of well-organized, but not yet solid, corpora lutea. There is little haemorrhage into the follicle at the time of rupture. The theca interna cells do not contribute any glandular elements to the corpus luteum. At the time of delivery the corpus luteum shows early degenerative changes, and large droplets of accumulated fat are present in the luteal cells. The latter are now smaller, and infiltration of the corpus luteum by leucocytes is marked. The stage of degeneration is reached several days earlier in pseudo-pregnancy. There is little difference between the corpora albicantia of a lactating and a pseudo-pregnant female. At the time of the next oestrus the corpus luteum has become greatly reduced and consists mostly of connective tissue in which shrunken luteal cells and fat globules can be found. By 3 months, when the pouch young are about to leave the mother, no trace can usually be found of the corpora lutea of pregnancy. It is suggested that functioning corpora lutea have no connexion with lactation, and that the corpora lutea do not determine the length of the cycle. As the opossum female does not come on heat during lactation, when there is no functioning corpus luteum, it appears that oestrus is inhibited, directly or indirectly, by lactation.

IV. THE EUTHERIAN CORPUS LUTEUM

Diagrams of different forms of developing corpora lutea are shown in Fig. 1.

(1) *Insectivora*

In the shrew, *Blarina brevicauda* (Pearson, 1944), the ovum in the mature follicle is surrounded by many layers of granulosa cells whose size and organization cause the follicle to resemble a corpus luteum. A large tuft of granulosa cells often projects from the follicle immediately after ovulation. Lutealization of the granulosa cells, and in some cases penetration of the ovum by the sperm, occurs before ovulation. The early corpus luteum is scarcely larger than the follicle from which it developed (0·5–0·55 mm.). The peripheral cells of the corpus luteum show the changes of lutealization first, and by the time of implantation the luteal cells are all equal in size. The corpus luteum reaches its maximum diameter (0·75 mm.) when the embryos are 3 mm. in length. The luteal cells are now large, their cytoplasm is granular and may show a peripheral clear area. Degenerative changes begin to appear at this stage, and are mirrored by an increase in the number of interstitial cells, a decrease in the blood supply, and by the presence of large follicles (0·5 mm.) in the ovary. The decline of the gland is rapid after the embryos have reached the 7 mm. stage. By the 10 mm. stage the gland has shrunk markedly, but many of the individual luteal cells are larger than at any previous time. Other luteal cells are highly vacuolated or present an empty appearance. The interstitial cells have increased in number, and the corpus luteum is less sharply demarcated by the thecal layers from the rest of the ovary. At the time of parturition the corpus luteum has practically vanished and can seldom be distinguished after a few days.

There is no infolding of the wall of the ruptured follicle in the common British shrew, *Sorex araneus* (Brambell, 1935). The antrum of the collapsed follicle disappears at the 8-cell stage except for a persisting central blood clot. The corpus luteum is fully developed by the time the blastocysts reach the uterus. Up to this time there has been an ingrowth of connective tissue until a complete network has been formed about the luteal cells and the central clot has been replaced. The cytoplasm of the luteal cells becomes vacuolated half-way through pregnancy, and by the end the corpus luteum is small, shrunken and distorted. It consists of a connective tissue reticulum with a few luteal cells in the interstices. The luteal cells disappear entirely a few days after parturition. In many retrogressing corpora, mitotic figures were observed in the luteal cells. In the pigmy shrew, *S. minutus* (Brambell & Hall, 1936), the corpus luteum is well formed at the 8–16-cell stage and remains at a diameter of 360–450μ throughout the greater part of gestation. There is a tendency for the gland to increase in size as gestation proceeds, but a rapid

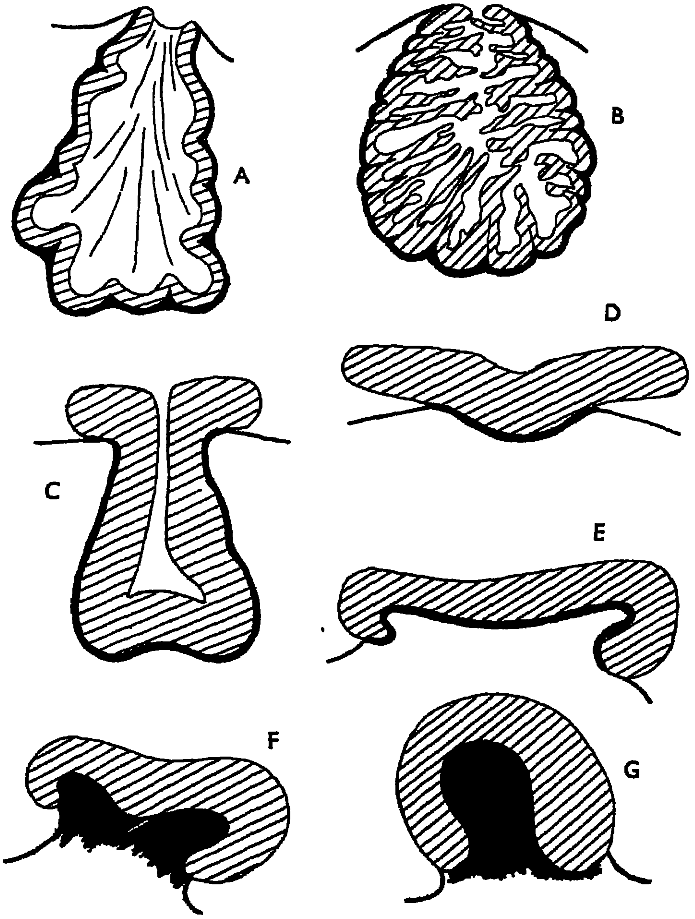

Fig. 1. Diagrams of developing corpora lutea (after Corner, 1943*a*). A. Usual form of early corpus luteum. B. Early corpus luteum showing marked folding of the mural epithelium, occasional in the sow (Corner, 1919), the bitch (Evans & Cole, 1931), the mare (Harrison, 1946), and the ca'aing whale (Harrison, 1947). C. Partial extrusion and eversion of corpus luteum in the rhesus monkey (Corner, 1943*a*). D. Marked eversion of corpus luteum in the rhesus monkey (Corner, 1943*a*). E. Extreme eversion of corpus luteum in the fox (Pearson & Enders, 1943). F. Everted and pedunculated corpus luteum in *Elephantulus* (van der Horst & Gillman, 1942), and the *Centetidae* (Strauss, 1938–9*a*). G. Horseshoe-shaped corpus luteum of *Elephantulus* (van der Horst & Gillman, 1942).

decline occurs towards the end, and it is probable that the gland disappears soon after parturition.

The granulosa cells of the corpus luteum of ovulation in the hedgehog, *Erinaceus europaeus* (Deanesly, 1934), do not appear to become lutealized. The granulosa cells are shrunken in comparison with those of the ripe follicle, and the whole corpus luteum of ovulation is much less conspicuous than the recently ruptured follicle. The gland contracts steadily up to the time of the next ovulation, and afterwards remains stationary. There is no definite histological difference between the corpora of pseudo-pregnancy and pregnancy. The granulosa cells hardly increase in size, although the nuclei may swell slightly. There is no apparent shrinkage or retrogressive change in the corpus luteum at the end of the pseudo-pregnancy cycle or just after parturition. The corpora lutea begin to retrogress during lactation. Fusion of corpora lutea is reported.

Lutealization of the granulosa cells in the mole, *Talpa europea*, commences before ovulation (Popoff, 1911; Mathews, 1935). The theca interna cells are said to take part in the formation of the corpus luteum, but their exact distribution is not described. The whole gland becomes highly vascularized a few days before parturition. Atretic corpora may be formed during lactation, but usually the corpora disappear soon after parturition.

The corpus luteum of the African jumping shrew, *Elephantulus myurus jamesoni*, has been thoroughly investigated by van der Horst & Gillman (1940*a*, *b*, 1942, 1946). The gland starts to develop before ovulation, and may even be partially responsible for rupture of the follicle. The ovum is pushed to the periphery of the follicle by the enlarging, elongated granulosa cells which soon obliterate the central cavity. There is no small stigma, but a cap of overlying germinal epithelium is torn off at ovulation, and almost half the circumference of the follicle is destroyed. The granulosa cells are thus everted into the periovarian space.

The theca interna reaches its maximum development just before ovulation, and is mainly restricted to the region farthest from the surface of the developing corpus. After ovulation the theca interna cells form a central core or 'cushion' to the everted corpus luteum, and sometimes a pedicle is formed. The thecal core is obliterated by the growing luteal tissue by the time the endometrium has reached the early dense stroma stage. The theca interna cells do not become theca-luteal cells, and it is suggested that their ultimate fate is to be metamorphosed into endothelial or reticular cells and thus to provide part of the connective tissue matrix of the gland. Later, in the early dense stroma stage of the endometrium, a well-marked capsular plexus develops, being most prominent at the pole of the corpus luteum opposite the thecal core. It is suggested that the development of the prominent part of the capillary plexus is due to a partial inversion of the corpus luteum at this stage. The capillary network that eventually forms throughout the gland differs from that found in many other animals in that it does not enclose individual cells, but divides the luteal tissue into groups of cells, thus giving the gland a trabeculated appearance.

The luteal cells are largest at the time of ovulation, and thereafter, as the corpus luteum sinks into the ovary, the cells decrease in size. At first the luteal tissue consists of an outer compact zone and a more loosely arranged inner zone, the luteal cells having a finely granular 'sodden' appearance. By the time the majority of the ova have moved into the uterine tube the luteal cells have lost their sodden appearance, and many of the cells in the compact peripheral layer have a vacuolated cytoplasm. In the early dense stroma stage of the endometrium most of the luteal cells lose their vacuoles, which are replaced by finely granular cytoplasm. The luteal cells shrink during this stage and their nuclei become more chromatic. By the early pre-polyp stage a number of small vacuoles are present in the luteal cytoplasm. Numerous discrete eosinophilic granules are found in the cytoplasm, as well as 'inclusion bodies' one-third of the size of the nucleus. Luteal cells with two nuclei are now commonly found. When the polyp is fully formed pyknotic and fragmented nuclei are present in the luteal cells which are now much smaller and contain more inclusion bodies. During the period of menstruation the luteal cells are arranged in groups of 8–12 cells. Their cytoplasm loses its vacuoles and is intensely eosinophilic; degeneration of the gland continues until a pseudo-capsule alone marks its original site. Fusion of two or more corpora occasionally occurs after they have become embedded in the ovarian stroma.

The corpus luteum of pregnancy develops in the same manner as that of menstruation as far as the stage related to the dense stroma and thickened epithelial stage of the endometrium. Three periods in the evolution of the gland can be recognized. The first extends from the time of ovulation until the embryo is 10 mm. in length. There is a gradual increase in size of the corpus luteum during this time, due solely to an enlargement of the individual cells. Two types of luteal cell are found, one with eosinophilic granular cytoplasm, the second with small vacuoles enclosed by strands of granular cytoplasm. The second period terminates when the embryo is 20 mm. in length, and during this time a large fat vacuole appears and disappears in turn from every luteal cell. During the third period, which covers the remainder of pregnancy, there is an initial increase in the size of the corpus luteum, due to further enlargement of the luteal cells. Small vacuoles are present during the first part of this third period, but they disappear progressively and the cell becomes finely granular. Thereafter the corpus luteum decreases remarkably in size, degeneration of the luteal cells occurs, and 4 days after parturition a corpus albicans is already formed.

Van der Horst & Gillman suggest that during the second period of luteal development in pregnancy a stage of readjustment is occurring. Although ovariectomies have not yet been performed, the fact that *Elephantulus* is liable to abort during this period tends to support this suggestion. In their opinion the period of readjustment may be associated with 'the stabilization of the placenta, following on an extensive destruction of the decidual cells in the uterine wall, and a remarkable modification in the main arterial circulation of the placenta'.

Follicular growth in the tenrecs, *Centetidae* (Strauss, 1938–9 *a*, *b*) is initially similar to that found in other Eutheria, but no cavity is found in the mature follicle.

The granulosa cells swell before ovulation and occlude the central cavity. Fertilization is said to occur while the ovum is still within the follicle, the sperm having penetrated the theca and spongy granulosa. The theca recedes at the top of the follicle and the ovum is extruded by a swelling out of the granulosa cells. The latter then become extruded themselves, and an everted fungiform corpus luteum is formed. This is connected to the ovarian stroma by a 'Thecabecher', similar to the thecal core found in *Elephantulus*. Lutealization of the granulosa cells takes place from the thecal core outwards. The changes occurring in the corpus luteum have been graded by Strauss into phases of proliferation, vascularization, maturity and retrogression. He is of the opinion that the theca interna cells form theca-luteal cells at the periphery of the early corpus luteum.

(2) *Chiroptera*

The early work of van der Stricht (1901 *a*, *b*, 1912) on the corpus luteum of the European bats has not yet been followed up with as much investigation as the unusual reproductive phenomena found in other species necessitates (see Wimsatt, 1944). Since the work of van der Stricht there have been brief descriptions of the corpora lutea of the British Horseshoe bats by Mathews (1937–8), some African bats (Mathews, 1941) and the American hibernating bat *Myotis l. lucifugus* (Wimsatt, 1944).

In *Nyctalus noctula* and *Plecotus aurita* the theca interna cells increase in size and become granular just after ovulation, at which time they can be seen distributed in the theca externa. At the time of ovulation the central cavity is reduced to a minimum. There is some evidence for the formation of tertiary liquor (van der Stricht, 1901 *a*, *b*, 1912). The theca interna cells are said to change gradually in appearance until they cannot be distinguished from the lutealizing granulosa cells. Van der Stricht believes that the increase in size of the corpus luteum is due to the increase in diameter of the blood vessels and lymphatics, to the increase in the amount of connective tissue, to the accumulation of fat granules in the luteal cells and also to the extension of the luteal tissue into the surrounding stroma. As gestation progresses the theca externa capsule disappears and the luteal cells are free to pass into the stroma of the ovary. From the time of implantation of the blastocyst to the end of gestation the luteal cells contain lipid. Throughout gestation the luteal cells shrink in size steadily, without, however, any appreciable changes of degeneration. Some evidence is given for the lutealization of the granulosa cells before ovulation.

In *Rhinolophus hipposideros* (Mathews, 1937–8) the corpus luteum is 500–550μ in diameter, and is nearly as large as the rest of the ovary by the time the blastocyst has reached the uterus. The main bulk of the corpus luteum projects from the ovarian surface and is attached to the ovary by a small pedicle. At ovulation the follicle is everted, and there is later a small ingrowth of connective tissue from the pedicle. Eversion occasionally occurs in *R. ferrum-equinum*, but the corpus luteum is usually found enclosed in the ovary. The corpus luteum is resorbed during lactation, and by the end has completely disappeared. However, in *Nycteris luteola* the corpus luteum has disappeared early in gestation, approximately at a time when the foetus does not exceed a C.R. measurement of 12 mm. (Mathews, 1941).

Differentiation of a theca interna and externa does not occur in *Myotis lucifugus* or *Eptesicus fuscus* (Wimsatt, 1944). Preovulatory hypertrophy of the granulosa cells is found in both these species. Ovulation is not followed by marked folding of the follicular wall. In *Myotis*, unlike *Eptesicus*, a central cavity rarely persists after ovulation, and in most cases the corpus luteum becomes solid even before the first cleavage spindle appears in the tubal ovum. A viscous tertiary liquor is present, which helps to plug the rupture point. The latter is often completely overgrown by connective tissue and germinal epithelium while the ovum is still in the 2-cell stage. The appearance of progestational reactions in the uteri of some specimens of *Myotis*, which are about to ovulate, suggests that progesterone may be produced in the last stages before follicular rupture.

(3) *Carnivora*

Since the patterns of the reproductive cycle differ greatly in the Carnivora, variations in the development of the corpus luteum are to be expected. Apart from the cat, however, detailed descriptions are scanty and further study is needed, especially in those *Mustelidae* in which implantation is delayed.

In the earliest ruptured follicles of the cat (Dawson, 1941) the wall is deeply plicated, the folds also involving the theca interna. No haemorrhage occurs into the collapsed follicle, and its contents are of a semi-gelatinous nature reminiscent of the tertiary liquor recognized by Robinson (1918) in the ferret. The ruptured follicle in the cat, however, never becomes markedly redistended after ovulation. On the 3rd day the wall of the follicle is markedly thickened and the plications are compressed against each other. The opening to the surface is lost on the 5th day, and by the 7th day the central cavity is almost obliterated. After the 7th day the plications are no longer visible, but connective tissue septa mark their original site as late as the 21st day. The corpus luteum reaches its maximum size 10–16 days after mating, so that its period of growth coincides with the period of endometrial transformation prior to implantation (Dawson & Kosters, 1944). After 20 days the corpora lutea of pregnancy start to retrogress, whereas retrogression is said to occur after 28 days in corpora lutea of pseudo-pregnancy (Liche, 1939). Van Dyke & Li (1938) find that the corpus luteum of pseudo-pregnancy is no longer active 20 days after ovulation. Incompletely separated corpora lutea are frequently found, and also mature follicles, showing no signs of atresia, may be seen included within fully developed corpora lutea.

There is local hypertrophy of the theca interna cells during oestrus and in early stages of post-coital activity, especially in the region of the cumulus (Dawson & Friedgood, 1940). The hypertrophied region is not recognizable 14–18 hr. after mating, probably due to the marked intra-follicular pressure. After ovulation many of the theca interna cells lose their fibroblastic form and become rounded or polyhedral. There is an initial migration of theca interna cells and endothelial cells into the mural granulosa 48 hr. after ovulation, completely penetrating the wall of the corpus luteum by the end of the 3rd day. Mitoses are common in the theca interna from the 36th hr. to the 7th day, but are infrequent in the granulosa cells. The theca interna cells are said to lose their rounded appearance after the 3rd day and again resume a fibroblastic form. They migrate between the lutealizing granulosa cells towards the lumen, where they lay down collagenous tissue. Groups of theca interna cells remain at the periphery of the gland, but there is no evidence for their development into theca luteal cells.

The pattern of the lutealizing granulosa cells is at first reticulate, but by the 7th day the cells have attained maximal size and are compactly arranged. They now have an inner perinuclear homogeneous endoplasm and an outer vacuolated exoplasm. The increase in size of the gland up to the 16th day is due to increase in the interstitial tissue and in the degree of vascularization. At the 16th day the peripheral vacuolation disappears from many of the luteal cells, and the cytoplasm appears 'fibrous', and the cell outlines are less distinct. By the 27th day the entire gland has become modified in this manner and there is an associated early decrease in its size. By the 50th day, however, the luteal cells have again become highly vacuolated and this fatty infiltration of the cells is regarded as evidence of degeneration. During the last days of pregnancy Dawson & Kosters (1944) find that the luteal cells contain numbers of small, refractile, acidophilic granules, sometimes in a perinuclear position, but frequently scattered throughout the cytoplasm.

The changes in the luteal cells between the 16th and 50th day have not been correlated with any functional change in the corpus luteum. During this period, except from the 15th–17th day, ovariectomy is invariably followed by abortion (Courrier & Gros, 1935, 1936), and thus the corpus luteum is apparently necessary for the maintenance of pregnancy during this time.

The corpora lutea of lactating animals are of a bright pink colour due to their increased vascularity following parturition (Dawson, 1946). The smaller vacuoles in the luteal cells disappear rapidly, some of the 'giant' vacuoles, however, persist. The functional capacity of this 'rejuvenated' corpus luteum is not known, but it is suggested that the phenomenon is either a secondary effect of released pituitary prolactin (or luteotrophin), or due to a general depletion of fat reserves in response to the needs of lactation. These changes in the corpus luteum are found only in lactating animals. In females, from which the kittens had been removed at birth, the luteal cells gradually decrease in size, whilst the vacuolation persists. It is, therefore, suggested that lactation may prolong the life of the corpus luteum in the cat.

The life-span of the corpus luteum may be as long as 6–8 months from the time of mating (Dawson, 1946). The luteal tissue is reduced by progressive cytolysis, usually not accompanied by leucocytic infiltration. The brilliant acidophil granules, present in late pregnancy and during the first weeks of the post-partum period, are absent during this later period. The small blood vessels, including the capillaries, have reduced lumina and are invested by a varying number of adventitial cells. These cells may be derived from the capsular region, or may be detached endothelial cells.

The corpora lutea of the wild cat, *Felis sylvestris*, are also slow to retrogress, and large corpora from the first heat persist when mature follicles are present at the second heat (Mathews, 1941–2).

Marked folding of the granulosa layer is present even in the mature follicle of the American fox, *Vulpes fulva* (Pearson & Enders, 1943). The folds increase in complexity as the follicle matures and each fold contains a large blood vessel. Small vessels extend from the large central one out into the spaces between the granulosa cells, and thus even before ovulation the granulosa cells have a rich blood supply. Due to this marked pre-ovulatory folding two main types of corpus luteum can be distinguished. In one type the appearance of a one-day-old corpus luteum is of an open 'lace-work' character, the folding of the mural granulosa having proceeded so rapidly that the cells are loosely arranged. In the second type the changes of lutealization have progressed enough to give a 'compact' appearance to the early developing corpus luteum.

The folding of the granulosa layer in the mature follicle is even more marked in the bitch (Evans & Cole, 1931; Mulligan, 1942). All the mural granulosa is thrown into complicated folds, each containing delicate connective tissue and blood vessels from the theca interna plexus. The early corpus luteum of the bitch usually shows a marked 'lace-work' appearance for the first few days, but it is more consolidated by the 8th day and is compact by the 18th day. The luteal cells reach their maximum size by the 10th day of metoestrum, or 16 days after ovulation. Degeneration of the gland probably commences on the 30th day of metoestrum, as judged by the condition of the endometrium, and many of the luteal cells show the presence of fat. The corpus luteum of pregnancy retains the same morphology at gestation as it had 3 weeks after ovulation, except that the central cavity becomes filled with connective tissue and blood vessels.

The degree of persistence of the central cavity in the ferret, *Mustela furo*, is said by Robinson (1918) to depend on the degree of separation of the internal limiting membrane at ovulation. The point of rupture of the follicle is closed by a tenacious coagulum, the tertiary liquor folliculi, which also redistends the collapsed follicle often to the size it originally was before ovulation. As the enlargement proceeds the coagulum of tertiary liquor breaks down into a granular detritus. Haemorrhage does not occur into the follicle at ovulation, but occasionally takes place after the redistension is completed. The corpus luteum reaches its greatest size 3–5 weeks after ovulation and the ovaries consist almost entirely of luteal tissue. The luteal cells show little or slight change until the end of pregnancy or pseudo-pregnancy, for the gland appears to atrophy only 3–4 days before birth of the young (Hammond & Marshall, 1930). There is no corpus luteum of lactation.

The corpora lutea of infertile cycles in the stoat, *M. erminea*, are smaller than those of pregnancy (Deanesly, 1935). The corpora lutea of ovulation are well vascularized, but the luteal cells remain small. Wright (1942), see also Watzka (1940), suggests that the so-called sterile matings with their smaller corpora lutea are indicative of delayed implantation. The corpora lutea of the weasels, *M. frenata* and *M. cicognani*, and of the martens, *Martes americana* and *M. caurina*, are small and inconspicuous when associated with unimplanted blastocysts (Wright, 1942). In the martens highly vacuolated luteal cells, comparable to those of the cat in late pregnancy and to those found in the Armadillo in similar circumstances, are present during the long period in which blastocysts remain unimplanted in the uterus. Fatty infiltration of the luteal cells may therefore be associated with temporary cessation of secretory activity, but is not necessarily indicative of irreversible retrogressive changes in the above circumstances. It is suggested by Wright that the corpus luteum in the marten undergoes some regenerative change, or is replaced by new or accessory corpora lutea before implantation occurs.

(4) *Pinnipedia*

New corpora lutea in the seal, *Callorhinus ursinus*, may be lobulated or elongated ovals (Enders, Pearson & Pearson, 1946). The corpus luteum later becomes spherical. A distinct antrum, at first fluid filled, later becomes invaded by luteal and connective tissue. Just before whelping the luteal cells contain vacuoles, which are still prominent when the corpus luteum begins to be resorbed a few days after whelping. The gland is larger when 1 year old than at 2–3 months. It persists for longer than a year, and takes more than 2 months after whelping to degenerate. Apparently the corpus luteum is unique in the seal in selectively suppressing follicular growth in the ovary in which it lies from within a few weeks of ovulation until several months after parturition during the following year. It is suggested that this may be due either to the relatively large size of the corpus luteum, which may be as large as the rest of the ovary, or to the subthreshold level of the follicular stimulating hormone because of the growth and distortion of the pregnant uterine horn.

(5) *Cetacea*

For a recent review of the literature dealing with the reproductive organs in the whalebone whales reference should be made to Mackintosh (1946). The fully developed corpus luteum in the whale is the largest known, and may reach a diameter of 10 cm. (Mackintosh & Wheeler, 1929). The corpus luteum of the false killer whale, *Pseudorca crassidens*, has been briefly described by Comrie & Adam (1937–8). An interesting feature of the corpus luteum in the whales is its persistence for many years, probably thoughout the life of the whale, in an inactive and degenerated condition (Wheeler, 1930). Thus the number of old corpora in one pair of ovaries may vary from one to over fifty. The difference in number is an indication of the number of ovulations that have occurred and thus of the age of a particular individual.

Harrison (1947) found active corpora lutea in the ovaries of six mature females from a school of ca'aing whales, *Globicephala melæna*, killed off the Faroe islands. The earliest specimen measured 2 cm. in diameter and showed evidence of recent rupture. The corpus luteum, however, that was found in a female pregnant with a 42 cm. foetus measured 4·5 cm. in diameter. The early specimens have their granulosa cells arranged in a loose 'lace-like' manner and the cells are small. There is considerable plication of the mural epithelium and theca interna cells are distributed about the periphery of the developing gland, and are also carried into the bases of the folds. The theca interna cells are large, polyhedral, and extensive vacuolation is present in their cytoplasm. In the later specimens the luteal cells are large, with an average diameter of 30μ and extensive peripheral vacuolation of the cytoplasm is found. A noteworthy characteristic is the presence of numbers of multi-nucleate 'giant' cells. These cells measure up to 45μ in diameter, and may contain as many as six nuclei. Towards the end of pregnancy the luteal cells lose much of their vacuolation and shrink in size. The amount of connective tissue increases greatly, and the vascularity of the gland is diminished by compression of the blood vessels and by their absorption into the developing scar tissue. The presence of luteolipin is demonstrated in the retrogressing corpora lutea.

(6) *Xenarthra*

The corpus luteum in the armadillo, *Dasypus novemcinctus*, develops rapidly until the volume of the corpus may equal the rest of the ovarian tissue. The ovary appears as a small cap of cells sitting on the corpus (Hamlett, 1932, 1935). During the period of delay in the implantation of the blastocyst the corpus luteum is large and fully formed, but its secretory activity appears to be suppressed. Implantation is attended, and may even be preceded by the appearance in the luteal cells of secretory droplets, which give the peripheral cytoplasm a vacuolated appearance. The corpus luteum enlarges and becomes highly vascular at this time. Following parturition the corpus, which retrogresses from the middle of embryonic development, continues to degenerate during the next few months until it has been entirely replaced by connective tissue.

(7) *Lagomorpha*

The earlier researches of Sobotta (1897), Honoré (1900) and Cohn (1903) have been extended by Marshall (1925), Togari (1926) and Deanesly (1930). Knowledge of the earliest changes occurring after ovulation has been increased by the work of Walton & Hammond (1928), and by the cinemicrographic studies of Hill, Allen & Kramer (1935).

Ovulation occurs 10–10½ hr. after copulation, and considerable haemorrhage takes place into the ruptured follicle. The greater part of the liquor folliculi remains in the cavity, which does not collapse to any great extent. The theca interna cells contain fat in the form of fine granules in the early stages, and together with endothelial cells they migrate in among the granulosa cells 12 hr. after copulation. The theca interna reaches its maximum development 24 hr. after copulation. The cells rapidly lose their fat granules, and by 30 hr. after copulation the entire gland is practically fat free. By 55 hr. the central cavity is closed to the exterior, and by this time the theca interna cells can only be distinguished with difficulty. By 3 days an extensive system of capillaries, associated with a connective tissue network, is present. Vacuolation of the luteal cells is apparent at this time, and the luteal cells reach their maximum diameter by the 5th–8th day. There is still a central cavity present, but it is obliterated by the 15th day. Vacuolation of the luteal cells steadily increases until 31 days after copulation, at which time it is most marked. The corpus luteum is absorbed gradually after the end of pregnancy; lactation, however, speeds up retrogression. The corpus luteum of pseudo-pregnancy shows marked vacuolation on the 18th day after coitus, and thereafter appears to degenerate fairly rapidly.

Deanesly states that when the theca interna is most prominent it contains cells with large vesicular nuclei and others of a fibroblastic nature; both contain 'fatty cytoplasmic granules'. She maintains that the theca interna plays a part in establishing the vascular network. The cells are rapidly used up, and by 36 hr. after ovulation no trace of them can be seen.

The corpora lutea in the wild rabbit attain a size of 2·5–3 mm. in diameter during pregnancy (Allen, Brambell & Mills, 1947). They develop slowly and attain their maximum size at mid-pregnancy, and do not appreciably decrease in size until the last 2–3 days of gestation. Retrogression is rapid after parturition.

(8) *Rodentia*

Apart from the mouse, guinea-pig and rat, which are easily bred in the laboratory little detailed histological description is available for the corpora lutea of the many other species. There are, however, indications that the life history does not follow a constant pattern.

The corpus luteum increases steadily in size throughout pregnancy in the spermophile, *Citellus tridecemlineatus* (Volker, 1905; Drips, 1919). Lipid droplets appear in the cytoplasm of the luteal cells half-way through pregnancy. Thirty-five days after parturition the corpus luteum is larger than at any other time. Retrogression sets in 8 weeks after parturition. In the early corpora lutea of the woodchuck, *Marmota monax* (Rasmussen, 1918), the luteal cells contain fatty droplets, but these soon disappear in the corpora lutea of pregnancy. Towards the end of pregnancy, and after parturition, the lipid reappears. The theca interna cells appear to invade the lutealizing granulosa cells, but the ultimate fate of the former is unknown. The persistence of full-sized corpora lutea is noted for many weeks after parturition. On the other hand, in the squirrel, *Sciurus carolinensis*, the corpora lutea reach maximum size (1·0–1·3 mm.) rapidly, but they begin to retrogress half way through pregnancy, and are considerably shrunken before parturition occurs. The characteristics of lutealization are never very marked (Deanesly & Parkes, 1933). The corpus luteum of *Geomys bursarius*, which may be everted when young, consists of cords of luteal cells, separated by trabeculae of vascular connective tissue. The theca interna, which is so remarkably enlarged before ovulation, apparently takes no part in the formation of the corpus luteum. The luteal cells show signs of degeneration near full term and their atrophy is almost complete by the time involution of the uterus has progressed to a stage where the placental scars are no longer visible. The central cavity persists until degeneration takes place (Mossman, 1937). The corpus luteum of the golden hamster, *Cricetus auratus*, develops rapidly and soon becomes solid (Deanesly, 1938). That of ovulation retrogresses rapidly after reaching a maximum of 700μ at about the 3rd day. The luteal cells are only a little larger than the original granulosa cells and they appear to be non-functional. The corpus luteum of pseudo-pregnancy is larger (820–860μ) and is better vascularized; signs of retrogression appear later, after about the 7th day. The corpus luteum of pregnancy is larger still (900–1000μ); retrogression sets in rapidly after parturition. Rapid retrogression of the corpus luteum of ovulation is also noted in the Chinese hamster, *Cricetulus griseus* after 2 days of dioestrum (Parkes, 1931). In the field vole, *Microtus agrestis* (Brambell & Hall, 1939), the corpus luteum continues to increase in size slowly throughout gestation reaching a maximum size of 1·25 mm. just before parturition. After rapidly attaining a diameter of 1·3 mm. at the 8-cell stage the corpora lutea of the bank vole, *Clethrionomys glareolus*, increase no further in size until implantation occurs (Brambell & Rowlands, 1936). After implantation a second slower growth phase begins and continues until parturition.

The classical work of Sobotta (1895, 1896) on the corpus luteum of the mouse has been followed by investigations by Allen (1922), Togari (1923, 1924) and Deanesly (1930). Sobotta found that the recently ruptured follicle became redistended with liquor in the manner that Robinson (1918) described in the ferret. Deanesly, however, describes all the corpora lutea obtained 4½ hr. after copulation as being solid throughout. Both Sobotta and Allen find remnants of a central cavity until 3 days after ovulation. All the authors agree that the theca interna cells proliferate actively during the first 24 hr., many of the cells are laden with fat and their nuclei are often vesicular and exceed those of the luteal cells in size. The theca interna cells are found invading the luteal tissue, mainly in relation to the capillaries. Bullough (1946) finds mitoses in the granulosa cells just after ovulation. The granulosa cells do not enlarge much at first, the fine osmicated granules found in these cells in the mature follicles are present until 10 hr. after ovulation. Capillaries spread to the centre of the gland by the 16th hr. after ovulation, and at this time the theca interna cells lose their fat. The corpus luteum reaches its maximum size 2–3 days after ovulation, the luteal cells are then at their largest, the peripheral cells contain fine osmicated granules, and lipids are present throughout the whole gland. Allen, however, states that the corpus luteum reaches its maximum size 10–14 days after ovulation, and there is then an equal proportion of luteal tissue to connective tissue. The theca interna cells do not form theca-luteal cells, and generally no trace of the theca interna cells can be found 60 hr. after ovulation. Deanesly and Togari are of the opinion that the ultimate fate of the theca interna cells is to give rise to fibroblasts which constitute both the walls of the blood spaces and the supporting tissue of the corpus luteum, and Deanesly suggests that these cells show affinities to the histiocytes of connective tissue.

Contrary to the observations of Long & Evans (1922), Boling (1942) finds that the average volume of the most recently formed set of corpora lutea in the rat increases rapidly between 12 and 48 hr. after the beginning of heat. There is little change in volume from the 48th to the 96th hr. However, during the first 12 hr. following the onset of the first heat subsequent to their formation, the average volumes of the corpora lutea decrease rapidly. Further retrogression continues at a slower rate. At no time was more than a single set of corpora lutea of maximum size observed in the ovaries of animals which had exhibited only 4–6-day cycles. Apparently corpora lutea formed during an 11-day cycle remain large during a successive 6-day cycle, and can thereby cause confusion. Greep (1938) observes that the injection of lutealizing hormones causes a rapid retrogression of persisting corpora lutea in hypophysectomized rats, which suggests that the factor causing the formation of the first set of corpora lutea may also be responsible for the regression of the 4-day-old corpora lutea which occurs subsequent to the next oestrus.

Vacuolation is observed in the luteal cells when the corpora lutea are 3½ days old, but at the 72nd hr. vacuolation is only noticed in association with the occurrence of small areas of more densely staining cells. Vacuoles are found until the corpora lutea are 4½ days old, but they are not, at this stage, as large as those found at 3½ days. Small vacuoles are numerous during the later stages of involution.

Everett (1945) finds that the increase of visible fat in normal corpora lutea does not progress uniformly, but increases gradually during dioestrum, reaching a temporary maximum in the late interval. During pro-oestrum a transient diminution occurs; the lipids reappear in abundance, however, during the next 24 hr. Some patches of fatty necrosis appear during pro-oestrum, coinciding with loss of fat in the parenchyma. The corpora lutea of the DA strain are deficient in lipid after mid-oestrum. No reduction occurs during pro-oestrum, nor are there patches of fat necrosis. Everett believes the lipids visualized to be mostly cholesterol and its esters, and that the deposition during oestrus of large amounts of cholesterol in the next youngest set of corpora lutea reflects some degree of luteotrophic stimulation during the preceding days. He suggests that there is strong evidence for cholesterol serving as a precursor of progesterone. It is also contended that the corpora lutea of the normal rat are moderately active during the cycle, contrary to general belief (Astwood, 1941). Dempsey & Bassett (1943) have demonstrated an accumulation of sterol-like material beginning in mid-dioestrum and reaching a maximum during the subsequent oestrus.

Long & Evans (1922) have pointed out that the corpora lutea of pregnancy cannot be distinguished from those of ovulation until about the 10th day of gestation. In the albino rat (Weichert & Schurgast, 1942) the corpora lutea of pseudo-pregnancy (1·517 mm.) are slightly larger than those of ovulation (1·37 mm.). The corpora lutea of pregnancy remain constant in size (1·45 mm.) until the 10th–11th day when they rapidly increase in size to 2·0 mm., and remain at that size from the 16th day until parturition. The corpora lutea of lactation average 1·589 mm. in diameter and the size is not influenced by the number of suckling young. The increase in size of the corpora lutea in prolonged gestation is comparable to the condition in normal pregnancy, except that because of the later implantation the gestation period is lengthened and the increase in size of the corpus luteum is correspondingly delayed. This delay is in general correlated with the number of suckling young.

The changes in character and distribution of the blood vessels in the corpus luteum of the albino rat have been studied in detail by Bassett (1943). Shortly after rupture of a follicle the granulosa layer is invaded by vascular sprouts from an inner capillary wreath in the theca interna. The capillaries invading the growing corpus become transformed into typical sinusoids, which contain intravascular fixed macrophages, resembling the von Kupfer cells of the liver. The vascular pattern is fully developed by 96 hr. after the onset of heat, and presents a rich network of sinusoids with superficial and deep arterial afferents drained by superficial venules. Usually only one large venule drains the deep plexus of central sinuses. The characteristic pattern is retained during regression, although the whole structure becomes progressively shrunken. During the early stages of growth of the corpus luteum, both the walls of the inner wreath of capillaries and the newly formed sinusoids are very permeable to India ink particles. The permeability gradually decreases as the vascular bed matures, and practically ceases by the time the corpus luteum reaches maturity.

Determinations of adenosine triphosphatase activity of the luteal tissue during oestrus, dioestrum, pseudo-pregnancy, pregnancy and lactation (Biddulph, Meyer & McShan, 1946) provide results which suggest that the ATP system may be concerned with the lipid metabolism of the corpus luteum, and possibly with progesterone formation and release.

The most recent investigations on the development of the corpus luteum of the guinea-pig, *Cavia porcellus*, have been made by Schmidt (1942), and they confirm the earlier work of Loeb (1906*a*) and Sobotta (1906). After rupture of the follicle the antrum is open to the peritoneal cavity for a short time. Strands of elongated granulosa cells extend into the follicle on all sides, and occasionally eversion of the developing corpus luteum occurs. Repair is completed within 2 days of ovulation. The elongated granulosa cells then retract and give the wall of the corpus luteum a compact appearance. The theca interna cells can be recognized by their granular and spindle-like appearance. Both the theca interna and granulosa cells hypertrophy and the two types become indistinguishable. The corpus luteum increases rapidly in size until it is 10–11 days old, after which time retrogressive changes commence. Endothelial proliferation reaches a peak on the 3rd day, and capillaries penetrate the central core of connective tissue on the 5th day. Some mitoses are seen in the luteal cells during the 8th–12th day.

Lipid droplets in the luteal cells are at first minute and evenly distributed, but on the 12th day large uneven vacuoles are present. The peripheral cells have shrunken pyknotic nuclei, whereas the central cells remain granular until the end of the next oestrus. The blood vessels become congested during the next oestrus, and there is renewed proliferation among the endothelial cells. During the second cycle following the formation of the original corpora lutea the retrogressing gland becomes smaller, and when 31 days old it is less than half its previous maximum size. At 32–33 days the corpus luteum consists of a core of hyaline tissue surrounded by cells containing uniformly small vacuoles and yellow granules, and at 48 days after ovulation there remain only a few strands of cells with bright yellow granules.

The fate of the corpora lutea formed at the first postpartum ovulation both in non-pregnant and pregnant animals has been described by Nicol (1933, 1934).

In the tree porcupine, *Erithizon dorsatus* (Mossman, 1940), corpus luteum formation is associated with rapid lutealization of atretic follicles in the same ovary. Thus numbers of accessory corpora lutea are formed.

(9) *Artiodactyla*

The development of the corpus luteum in the sow has been fully described by Corner (1915, 1919, 1921); in the sheep by Marshall (1904), Grant (1934), Quinlan & Maré (1931) and Warbritton (1934); in the cow by McNutt (1924) and Hammond (1927); and in the goat by myself (Harrison, 1948). In the cow there is frequently a fluid-filled central cavity to the gland, which often persists throughout the life of the organ. In the sheep, sow and the goat, the corpus luteum is generally solid by the 8th day after ovulation. Herniation of the adult corpus luteum is commonly found in the cow, and is occasionally found in the sow and the sheep, but is rare in the goat. The maximum diameter of the corpus luteum is generally larger by 115–125 % than the mature follicle.

The histological changes that occur in the corpus luteum of non-pregnancy are similar in the four animals and in the red deer (Harrison, unpublished). Invasion of the granulosa cells by the theca interna cells, with associated disruption of the membrana propria, commences between the 1st and 3rd day after ovulation. Vascularization of the gland begins about the same time, and nearly every cell has an endothelial coat by the 12th day. Andersen (1926) has described the blood vessels of the corpus luteum in the sow in some detail. The granulosa cells are fully lutealized by the 5th–6th day, and peripheral vacuolation of the cytoplasm of the luteal cells is found progressively from the 2nd day. It appears that in the ovaries of the Artiodactyla the theca interna cells do not revert to fibroblasts. Solomons & Gatenby (1924) suggest that the reticulum found between the luteal cells is laid down by the theca interna cells. Corner (1919, 1920), however, has produced evidence showing that the reticulum is in all probability laid down by endothelial cells that have passed into the corpus luteum. Added support for this view is provided by observations on areas of the corpus luteum in the goat in which endothelial invasion, together with reticulum deposition, can be seen before the theca interna cells have reached the area (Harrison, 1948). The fate of the theca interna cells will probably not be finally settled until a selective staining method for these cells is developed. Corner (1944) has made a preliminary investigation of the distribution of the theca interna cells in the corpus luteum of the sow, using Gomori's method for the demonstration of alkaline phosphatase. He finds the theca interna cells, laden with phosphatase, interspersed among the granulosa cells at the 18th day of pregnancy. After this time the luteal cells also acquire granules of phosphatase and the differentiation of the theca interna cells is more difficult.

Retrogression of the corpus luteum in the non-pregnant animal commences at the 13th–16th day after ovulation. The degenerative process is mirrored by a marked peripheral vacuolation and cytoplasmic shrinkage in the luteal cells. This is succeeded by fragmentation of the nuclei and subsequent disappearance of the luteal cells. Corner (1921) finds numerous angular and elongated cells, with foamy cytoplasm, and a wealth of osmium-staining fatty material, which survive the degenerative process. He believes these cells to be remnants of the theca interna (see also Hammond, 1927). I have found similar remnants of degenerating luteal cells in the site of old corpora lutea in the goat (Harrison, 1948). Morica (1940) finds many reddish crystals highly birefringent, in atretic corpora lutea of the cow. Rossman (1942) and I myself (Harrison, 1948) were unable to find luteolipin in the retrogressing corpora lutea.

The corpus luteum of pregnancy remains about the same size as that seen on the 12th–14th day for the early part of pregnancy. Corner (1915) has differentiated a number of stages in the life of the gland in the sow. He divides the early developmental stages into a preparatory stage of 25 days, and then into two stages covering the next 15 days in which there is a progressive appearance of vacuoles in the exoplasm of the luteal cells. From the 40th to the 75th day he finds a transitional period in which the vacuoles disappear. From the 75th day onwards diverse forms of endoplasmic vacuolation occur, and retrogressive changes appear on the 110th day. In the goat there is also a preliminary stage lasting about 35 days during which the appearances of the corpus luteum resemble those seen in the corpus luteum of non-pregnancy at the 10th–12th day (Harrison, 1948). From the 35th to the 45th day there is a gradual shrinkage in size of the luteal cells. Besides luteal cells containing small vacuoles numbers of the luteal cells contain large single vacuoles in the cytoplasm. At the 45th day there are no large vacuoles, and the general appearances of the corpus luteum are the same as those found on the 20th day. From the 55th to the 60th day both the cytoplasm and the nuclei of the luteal cells undergo contraction.

It is suggested that the period extending from the 35th to the 45th day marks a 'transition period', or period of readjustment during which time the placenta becomes fully established. It is significant that in the sheep Grant (1934) notes an increase in the size of the corpus luteum from the 14th day, which is at the time when destruction of the uterine epithelium is commencing (Assheton, 1906). It is also from the 30th day onward into the second month of pregnancy that the foetal membranes become fairly firmly attached to the cotyledons by finger-like downgrowths that eat into the tissues of the cotyledon (Hammond, 1927; Assheton, 1906).

(10) *Perissodactyla*

The macroscopic appearances of the corpus luteum in the mare have been described by Kupfer (1928), and by Hammond & Wodzicki (1941). The latter finds that the active stage of the corpus luteum is short compared with that of the cow and the sow, and that the maximum diameter of the fully developed gland is below that of the mature ovarian follicle (see Aitken, 1927). I find that the corpus luteum reaches its maximum diameter by the 10th–14th day after ovulation (Harrison, 1946), which corresponds approximately to the time of implantation of the blastocyst (Hamilton & Day, 1945). At this time the luteal cells begin to show a vacuolated outer zone and an inner perinuclear homogeneous area. The theca interna cells pass through the disrupting membrana propria from the 2nd day onwards, and can be seen for about 7 days after ovulation. The theca cells gradually lose all the characteristics of those found in the 'thecal gland' before ovulation, and it is difficult to distinguish them after this time.

Two types of developing corpus luteum can be distinguished in the early stages. The commonest type presents the open 'lace-like' form, also observed by Cole, Howell & Hart (1931) in the mare. The cells are elongated and stretch out into the lumen of the collapsed follicle. The second type shows a more compact arrangement of the lutealizing granulosa cells. Vascularization of the gland is well advanced by the 4th–5th day. By the 14th day a fine network of reticulum can be seen about each individual cell by silver impregnation methods. The sites of the theca interna cells are marked by areas, heavily impregnated with silver, among the fine network surrounding the luteal cells. The trabeculation of the gland by processes of theca externa cells is a marked characteristic of the corpus luteum of the mare. The corpus luteum of the non-pregnant animal starts to degenerate at the 14th–17th day after ovulation, and gradually becomes darker in colour. Remnants of the corpus luteum can be seen for about 8 weeks after ovulation.

Four stages have been described in the corpus luteum of pregnancy by Cole *et al.* (1931). During the first 40 days there is only one corpus luteum in the ovary, which starts to degenerate towards the end of this period. About the 40th day several follicles become fully lutealized and give rise to multiple accessory corpora lutea. Some of these structures may not be true corpora lutea, but lutealized theca interna cells which remain after the granulosa cells have degenerated (Kimura & Lyons, 1937). Regression of the corpora lutea commences about the 150th day, and only minute vestiges of the glands are present during the later stages of pregnancy. It is noteworthy that ovariectomy may be performed at the 200th day without interruption of pregnancy (Hart & Cole, 1934).

(11) *Primates other than man*

In the howler monkey, *Alouatta palliata*, and the spider monkey, *Ateles geoffroyi*, there is little lutealization of the granulosa cells until a marked ingrowth of capillaries into the granulosa layer is noted (Dempsey, 1939). Fat deposition then occurs in the theca interna and granulosa cells, the latter eventually becoming larger than the thecal cells. The central cavity disappears late in the luteal phase. During the whole life time of the corpus luteum it is possible to distinguish the thecal and granulosa elements by their position and appearance. Eventually, the wall of the corpus luteum disappears so that the gland becomes indistinguishable from the interstitial tissue.

The corpus luteum of the macaque, *Macaca mulatta*, has been comprehensively described by Corner, Bartelmez & Hartman (1936), Hartman (1932), Corner (1940, 1942, 1945), and Rossman (1942). Prior to rupture the granulosa cells become loosened, spindle-shaped and radially arranged. Blood vessels begin to invade the granulosa cells on the 2nd day, and the granulosa cells present epithelioid characteristics by the 4th day. Blood vessels reach the inner part of the wall of the corpus luteum about the 4th day, and at this time capillary sprouts and fibroblast-like cells enter the central cavity. The latter cells are apparently derived from the endothelial cells. Venous channels develop in the inner part of the gland on the 6th day and organization may be considered complete about the 8th day. At this time the capillary network is uniformly distributed through the wall of the corpus luteum. The theca interna cells are not numerous in the mature follicle, and mitoses are infrequent in these cells after ovulation. On the 1st day after ovulation the theca interna cells may be recognized by virtue of their position, larger size, sharper outlines, more vesicular nuclei and lipid-filled cytoplasm. However, from the 4th to the 8th day there is little to distinguish the thecal cells from the lutealizing granulosa cells. Later, on the 10th day, the granulosa cells so exceed the thecal cells in size and the vacuoles of the luteal cells are so large and irregular that the granulosa cells may be distinguished clearly from the thecal cells.

In animals that do not become pregnant the first signs of degeneration are seen about the 13th day after ovulation. Large amounts of lipid material collect in the cells, followed by destruction of the nuclei, the latter sign always being present on the first day of the menstrual flow. Numerous cells can be seen having large numbers of closely packed vacuoles of uniform size evenly dispersed throughout the cytoplasm ('mulberry' cells). Some cells contain large round vacuoles, one or two to every cell. During menstruation almost every cell shows either the 'mulberry' type of vacuolation, or the 'giant' vacuoles. The volume of the corpus luteum in non-pregnant animals increases until a very few days before the beginning of menstruation and then decreases suddenly. It is suggested that the production of progesterone by the corpus luteum must cease not more than 3 days before the onset of menstruation.

The corpus luteum of the pregnant animal cannot be distinguished from that of non-pregnancy of the same age until visible degeneration of the latter sets in on the 13th day. From the 13th to the 19th day the corpus luteum of pregnancy remains approximately the same. By the 24th day there is a reduction in size of the luteal cells, together with a disappearance of the lipid vacuoles, and a consequent reduction in the proportion of cytoplasm to the nucleus. The network of capillaries is larger. The theca interna cells retain their lipid and can be seen as clumps of cells packed into the folds of the corpus, especially at the bases of the folds. Thus the period extending from the 19th to the 24th day is described as one of 'transition', which has been associated with the fact that gonadotrophic substances in the urine of early pregnancy are disappearing at this time (Hamlett, 1937). There may also be a connexion between the changes occurring in the corpus luteum at this time with the 'placental sign', which is seen some time between the 13th and 20th day (Hartman, 1929).

The corpus luteum of pregnancy after the third week can be distinguished by its distinctly folded pattern, the small size of the luteal cells and absence of lipid vacuoles, prominence of the peripheral capillary network, and the distinctness of the theca interna cells. This typical pregnancy state apparently persists until the 146th day of pregnancy. Specimens obtained on the 154th and 169th day show progressively advancing signs of degeneration.

Besides the typical manner of retrogression two atypical forms have been described by Corner, Bartelmez & Hartman (1936), Corner (1940, 1942). One of these, corpora aberrantia, appears to be a form of persistent corpus luteum, or lutealized follicle. 'Certain of the corpora lutea, after the period of bloom, come under some atypical influence, presumably from the pituitary, and instead of degenerating in the usual fashion pass into a state of prolonged existence resembling that of the corpus luteum of pregnancy (of 25th day).' This form is marked by distinct evidence of folding of the walls and the presence of theca interna cells about the border and at the bases of the folds. Corpora aberrantia persist for at least 15½ weeks, and as they grow older they lose their connexion with the ovarian surface. There is little change in the appearances of the epithelioid elements during the first 4 months of their existence. Their ultimate fate has not been determined, but it is suspected that they may survive for 23 weeks. Corpora aberrantia do not produce progesterone, and the fact that in one animal corpora aberrantia had retrogressed more rapidly than in others suggests that the persistence of this atypical form requires a stimulus from some external source, presumably the pituitary. The suggestion that the transformation from a normal corpus luteum to a corpus aberrens occurs during the earliest phase of retrogression is supported by the work of Rossman (1942) on the distribution of luteolipin.

A form of corpus luteum is also found as the result of lutealization of an unruptured follicle at the same time as the normal corpus forms. This type is called an accessory corpus luteum. The cells pass through the same series of changes as the normal corpus, but early forms may be distinguished by the presence of an undischarged degenerating ovum. Accessory corpora lutea are apparently formed in about 17% of ovulatory cycles. This form probably produces progesterone, but in the specimens described, the amount of luteal tissue is too small to have produced significant amounts of the hormone. It appears that if a corpus luteum becomes a corpus aberrens, all accessory corpora will become corpora aberrantia accessoria.

The distribution of lipin and luteolipin has been described in detail by Rossman (1942). In the standard corpus luteum of *Macaca* luteolipin may be detected at the time of flow, and traces at least are found post-menstrually. From 2 to 6 weeks, although the nucleus is relatively larger in relation to cell size, it is smaller than in the corpus of the bloom period. From 6 to 13 weeks many of the luteal cells are larger than in the preceding stage, but this increase is to be contrasted with evidence of shrinkage of the gland as a whole. Luteolipin, now widely distributed throughout the cell, may appear as spheroids up to 5μ in diameter. Homogeneous wreaths and rings of luteolipin can be seen surrounding lipin droplets. During the final stage of retrogression, 13th–17th week, luteolipin is the predominant cytoplasmic component, forming a dispersed phase in which the lipin droplets are embedded. Retrogression of the corpus luteum involves a slow process in which luteolipin accumulates in the cells to the partial or complete exclusion of the soluble lipin droplets. This accumulation of luteolipin enables the age of a retrogressing corpus luteum to be estimated with some accuracy.

Zuckerman & Parkes (1932) describe marked folding of the follicular wall in the baboons, *Papio porcarius* and *P. hamadryas*, after ovulation. This gives the corpus luteum a lobulated appearance. The luteal cells reach their maximum development 7–8 days after ovulation. These authors find that theca interna cells are used to form the vascular reticular system. In non-pregnant animals the luteal cells show signs of vacuolation soon after the time of maximum development. By the 13th day, when the menstrual flow commences, signs of retrogression are marked, and at the end of the menstrual flow on the 17th day the corpus luteum has assumed a fibrous appearance. By the time the next luteal phase is well established, the corpus luteum of the previous phase is hardly discernible. In pregnant animals the corpus luteum attains a larger size, and the luteal cells are individually larger than in the fully developed corpus of non-pregnancy. Maximum development occurs during the 3rd week of pregnancy, but the volume of the corpus luteum, and the size of the luteal cells falls after 4–5 weeks and remains fairly constant for at least 26 weeks. The corpus luteum disappears rapidly after parturition.

Culiner (1945, 1946) finds that the theca interna cells undergo changes similar to those of lutealization, both in atretic and cystic follicles. They may give rise to theca luteal cysts and 'yellow bodies', which resemble corpus luteum cysts and corpora lutea respectively. Culiner suggests that this precocious activity of the theca interna may be related to irregularities of the menstrual cycle and to abnormalities of the endometrial pattern. However, the author does not illustrate the appearance of the whole ovary from which his examples are derived, and it has yet to be demonstrated that a similar production of aberrant and accessory corpora lutea, such as have been so fully described in *Macaca mulatta* by Corner, is not occurring in the baboon ovary.

A brief description of the corpus luteum in the gorilla is given by Saglick (1938), who finds that the corpus luteum of pregnancy is like that of the same stage in man. Lutealization of the theca interna cells is seen, and the cells are abundant in the borders of the gland and in the in-growing trabeculae. Marked groups of thecal cells at the periphery of the corpus luteum of the chimpanzee, *Pan satyrus*, have been described by Corner (1945).

(12) *Homo*

For a review of the earlier work on the human corpus luteum the reader should consult the articles quoted in the Introduction. The review by Pratt (1935) is particularly recommended for its interesting historical approach. The paper by Meyer (1911) must still be considered the classical work on the human corpus luteum, and it is a fundamental statement upon which all subsequent work has been based. For work carried out during the last two decades reference should be made to Watrin (1924), Solomons & Gatenby (1924), Shaw (1925), Chydenius (1926), Horrenberger (1928), Allen, Pratt, Newell & Bland (1930), Meyer (1932), Joachimovits (1935), Portes, Ascheim & Robey (1938), Gillman & Stein (1941), Fraenkel, Buno & Grosso (1941), Brewer (1942), Geist & Russell (1942), and also to Greulich, Morris & Black (1943) for the ageing of the corpus luteum and the timing of ovulation.

Meyer found it convenient to divide the life history of the corpus luteum into four phases, now well known as the phases of proliferation or hyperaemia, vascularization, maturity or bloom, and retrogression. There is now little doubt that an initial proliferation of the granulosa cells does occur in the early human corpus luteum during the first day after ovulation. In the corpus luteum of menstruation there is a gradual increase in functional activity during the first 8 days, and a peak of activity is reached by the 8th–10th day, which covers the time at which the blastocyst is probably implanted. A definite reduction in activity can be recognized by the 10th–11th day. During the first 8 days the granulosa cells show little change from their appearances in the wall of the mature follicle. They remain in the form of definite groups of cells about the periphery of the gland and in the bases of the folds formed in the wall of the collapsed follicle. These cells have been designated by the term 'para-lutein' cells, or the whole group of cells has been collectively described as the 'para-lutein gland'. At the termination of the so-called stage of vascularization certain regressive changes appear. There is a decrease in the amount of blood in the vessels and regressive changes appear in the blood vessels themselves. Fatty degeneration and degeneration by simple atrophy take place in the luteal cells. There is a sharp increase in the amount of visible lipids in the luteal cells, and an increase is also found in the cholesterol esters accompanied by a diminution of the phospholipid content. Organization accompanies this degeneration, and the whole corpus luteum is replaced by a structureless hyaline body called the corpus albicans. It is suggested that complete organization of the corpus luteum takes between 7 and 10 months. Some degree of haemorrhage occurs into the cavity of the corpus luteum during the early part of the stage of retrogression.

The corpus luteum of pregnancy grows steadily in size until the 50th day, after which it increases rapidly until the 60th day. It then shrinks, due to the gradual obliteration of the central cavity and diminution in thickness of the surrounding fibrous tissue. This central cavity develops early and reaches a maximum size between the 50th and 60th day. The luteal tissue remains fairly constant throughout pregnancy. The theca interna cells are abundant in early pregnancy around the trabeculae and septa. They form an almost complete subcapsular rim of theca-luteal cells. They lack colloid droplets and vacuoles. They reach their greatest development at the 2nd–3rd month, and disappear shortly after the obliteration of the central cavity in the 5th month. The theca-luteal cells account for as much as 8% of the total luteal tissue at their time of greatest development.

It is suggested that increasing amounts of theca-luteal cells are added to the corpus luteum throughout its functional life. Lipid and secretory granules, the latter staining red with Mallory's stain, are most numerous in the granulosa cells during early pregnancy. 50% of cells contain these secretory granules in the early months, but the number falls to 2% towards the end of gestation. The lipid granules also decrease in amount towards term. Colloid droplets are found in few of the cells during the early months, but they increase steadily in amount through pregnancy, and at the same time chromidial substances diminish in amount. The secretion of the granulosa cells is said to appear as a non-stainable secretion in vacuoles which are more abundantly distributed in cells in the inner third of the luteal wall close to the fibrous layer separating the luteal cells from the central cavity. Stagnation of the contents of these vacuoles is suggested as the cause of the stainable colloid. Calcium salts may be deposited later in the colloid, and is probably a sign of further degeneration of the colloid material.

Since it is rare to find colloid droplets in the cells of the corpus luteum of menstruation it has been suggested that their presence may be used as evidence of pregnancy for medico-legal purposes. However, the colloid material is apparently very labile and the immediate acquisition of fresh material is essential for its demonstration. It has yet to be definitely proved that the corpus luteum of menstruation does not contain these colloid droplets. There is evidence that the period covering the 50th–60th day of pregnancy may be a 'critical' one, during which the corpus luteum undergoes changes that parallel the metabolic disturbances in the body. It is also suggested that involution of the corpus luteum may commence as early as the second month (Gillman & Stein, 1941).

V. DISCUSSION

It will be seen from this survey that it is now certain that the majority of the luteal cells of the corpus luteum are derived from the granulosa cells of the mature follicle.

The fate of the theca interna cells, however, is far less definite. These cells may be very poorly differentiated, as in some Chiroptera and Marsupalia. They may remain relatively unchanged in groups at the periphery of the gland, as in the Monotremata and in man, or they may invade the lutealizing granulosa cells as in many other species. Some authors believe that the theca interna cells disappear from view during the early post-ovulatory period. Others believe that they revert to fibroblasts and lay down the connective tissue matrix of the gland. In other species the theca interna cells persist among the lutealized granulosa cells and eventually cannot be distinguished from the latter. It thus appears that the fate of the theca interna cells varies greatly in even quite closely related species.

The problem is further complicated by a consideration of the functions of the theca interna cells. In the maturing follicle, the theca interna cells undergo proliferation and hypertrophy in many animals until the entire mass of cells is so large that the term 'thecal gland' has been applied to their maximal development at pro-oestrum (Mossman, 1937). There is evidence that the 'thecal gland' is responsible for the production of the majority of the oestrogens excreted by the ovary during pro-oestrum and oestrus (Corner, 1938). Thus, in considering the fate of the theca interna cells, it must be realized that they are originally derived from the cells of the ovarian stroma, and that they then assume a glandular form and are probably a source of oestrogen. It might be expected that the theca interna cells would cease to function during the postoestrous period, and in many animals there is definite evidence of a decline in activity at this time. Furthermore, in a number of species the theca interna cells cannot be recognized in the corpus luteum after a few days, and they may have disappeared entirely. However, it has been found that oestrogen prolongs the life of the corpus luteum in the hypophysectomized rabbit (Robson, 1937). Therefore it is possible that the theca interna cells continue to secrete oestrogen when they have been incorporated in the corpus luteum. Such speculations are purely presumptive until it can be demonstrated that the theca interna cells are actively secreting during this period.

Should the theca interna cells revert to fibroblasts, as so many authors have claimed, it appears that these cells enjoy a freedom of interconvertibility not possessed by other adult cellular types. Indeed, Corner (1945) has stated: 'To what extent such cell types may be interconvertible is a difficult question in general, and nowhere more so than in the ovary.' The theca interna cells may be capable of this range of activity and function, presumably under the control of some external factor, such as the pituitary hormone. However, there is evidence that in the Artiodactyla and in *Macaca* that the so-called fibroblasts in the maturing corpus luteum are not metaplastic theca interna cells, but are endothelial cells behaving in a manner reminiscent of the reticulo-endothelial cells in the liver. The final solution to the problem of the origin of the connective tissue of the corpus luteum may only be solved when specific histochemical staining methods for the theca interna cells have been developed, or when the knowledge of the factors controlling metaplasia and interconvertibility of embryonic and adult cell types is more advanced.

The corpus luteum is generally fully developed by the time of implantation of the blastocyst. If the ovum is not fertilized the corpus luteum shows degenerative changes soon after the time at which the blastocyst would have implanted had fertilization occurred (10–18 days after ovulation). In those species in which pseudo-pregnancy occurs the corpus luteum is often larger and persists for longer than that of the cycle.

Vacuolation of the luteal cells is one of the first signs of a retrogressing corpus luteum, but is not necessarily indicative of an irreversible process. Vacuoles appear in the luteal cells of animals in which delayed implantation occurs, and also during the period associated by many authors with the establishment of placental activity. It is not yet clear how the production of these lipid-containing vacuoles fits in with the biochemical changes that must occur during the production of progesterone.

Further work must be carried out to study the precise effects of implantation on the corpus luteum. It is probable that the developing trophoblast produces some chemical or hormonal substance that directly or indirectly has some action on the corpus luteum (Corner, 1938; Wislocki & Streeter, 1938; Allen *et al.* 1939). Any attempt to explain the factors responsible for the persistence of the corpus luteum after implantation can only be presumptive, until further experimental work has been performed. It must also be clearly understood that the life-span of the corpus luteum of pregnancy is not absolutely related to the length of gestation. Although in some species the corpus persists until parturition, and even into the lactation period, in many species the corpus luteum begins to retrogress during pregnancy. Asdell (1946) has suggested that the life of the corpus luteum is related to the prolactin level in the anterior pituitary. In future the developmental changes in the corpus luteum, which are now well established in many species, must be more closely related to the biochemical and hormonal changes occurring in the ovary, the blood, the urine and the pituitary of the pregnant female, and in the trophoblast and the placental circulation.

VI. SUMMARY

1. Published descriptions of the development of the corpus luteum show that the majority of the luteal cells are derived from the granulosa cells.

2. In the vertebrates, other than mammals, the development of a corpus luteum does not appear to be associated solely with viviparity.

3. The fate of the theca interna cells varies greatly, even in closely related species.

4. The theca interna cells may remain in groups at the periphery of the gland, or they may invade the developing corpus luteum and either: (*a*) disappear entirely a few days after ovulation, (*b*) become indistinguishable from the granulosa cells, or (*c*) possibly revert to fibroblasts.

5. The function of the theca interna or theca-luteal cells in the mature corpus luteum is unknown. It is, however, possible that they secrete oestrogen.

6. The blood vessels of the corpus luteum are derived from the theca interna plexus of capillaries; the corpus luteum is well vascularized by the time that implantation occurs.

7. The connective tissue of the corpus luteum is believed by many authors to be derived from theca interna cells that have reverted to fibroblasts. In the Artiodactyla and in *Macaca*, and possibly in other animals, there is evidence that the endothelial cells give rise to the reticular framework.

8. The corpus luteum is generally fully developed by the time of implantation of the blastocyst.

9. The appearance of the vacuoles in the luteal cells of pregnant mammals is not necessarily indicative of impending cessation of function or of degeneration. Such vacuolation may mark a 'resting period' as in those animals in which implantation is

delayed, or a 'transition period' during which the placenta is becoming established structurally and functionally.

10. The time of appearance of retrogressive changes in the corpus luteum of pregnancy in any species is probably related to the degree of activity of the placenta in that species and to the prolactin level in the anterior pituitary.

11. Accessory, aberrant and other atypical forms of corpora lutea have been reported in the macaque, the tree porcupine and the mare.

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ADDENDUM

While this article was in the press several noteworthy papers have appeared. Everett (1947) finds that cholesterol storage in the luteal cells of the rat depends on the ratio of LH/luteotrophin. LH appears to have the specific action of increasing the cholesterol stored in luteal tissue, whereas the degree of luteotrophic stimulation determines the rate at which cholesterol is utilized. On this basis an explanation is offered for the sequence of changes in cholesterol content of the corpus luteum of the cyclic rat already mentioned in §IV, 8. Further evidence is provided for the suggestion that cholesterol is a precursor of progesterone. Bassett (1948), using colchicine treated albino rats, finds that there is considerable mitotic activity throughout the early pregnancy corpora lutea. During the 4th to the 7th day of pregnancy there is an increase in the concentration of cells while the volume of the corpus luteum is not increasing. This suggests that there is an active proliferation of luteal cells or some precursor cells during the early period of development of the corpus luteum. However, the late volume increase is due to a luteal cell hypertrophy.

Höfliger (1947) in his investigations on the ovary of the cow, finds that the corpus luteum is fully developed 9 days after ovulation. The theca interna cells migrate among the granulosa cells in relation to the fibrous septa from the 6th day after ovulation. Retrogression of the corpus luteum of the cycle commences on the 14th day. The corpus luteum of pregnancy, which grows steadily during the first 3 months, cannot be distinguished from that of the cycle. The lipid content of the luteal cells increases slowly up to the 5th month, then more rapidly until the 7th month. Colloid inclusions are found in increasing amounts from the 3rd month. Amoroso, Hancock & Rowlands (1948) have confirmed the observations of Kimura

& Lyons (1937) that regression of the primary corpus luteum in the pregnant mare commences about the end of the 1st month. One or more ova were recovered from the uterine tube of nine mares which were examined post-mortem when 46–73 days in foal. The ovaries all contained one or more fully formed corpora lutea, but in none, with the exception of the mare examined on the 46th day, could the primary corpus luteum be recognized. It is probable that during the 2nd month of pregnancy a succession of follicles ovulate from which new corpora lutea are formed.

Dubreuil and Rivière (1946) in a general review of the morphology of the corpus luteum, which includes their own observations of 450 human ovaries, arrive at the conclusion that the corpus luteum secretes both progesterone and oestrogen. They believe that the period of activity of the luteal and theca interna elements is the same. They suggest that the progestational phase of the cycle should be termed the oestro-progestational phase for this reason. They list four main methods by which the human corpus luteum may degenerate: a fibro-hyalin method after which fibrous remnants may persist for a long time; a lipoid degeneration leaving a persisting yellow or orange body; a rapid necrobiosis, seen chiefly in the ovaries of young women; and a slow necrobiosis usually found in the corpus luteum of pregnancy. The authors are of the opinion that the only elements which take part in histolytic degeneration are the epithelial cells, the remaining cells are said to dedifferentiate to the type of cell found in the ovarian stroma. In a previous paper Dubreuil (1944) gives a list of specific characters which enable the corpus luteum of pregnancy to be differentiated from that of the cycle. In the corpus luteum of pregnancy he finds: a more complete vascularization with more numerous arterioles and venules; the presence of colloids; greater thickening of the glandular layer and a reduction in size of the central cavity; organization of the central coagulum; persistence of an active theca interna during the first few months; and deposition of lipid starting at the 5th month. However, these distinctions are all mainly of degree, and except for the presence of colloidal material in the luteal cells, the fallibility of which has already been discussed, there is still no certain histological method of stating whether a given early corpus luteum from the human ovary is one of the cycle or of pregnancy. The later stages of the corpus luteum of pregnancy can be more easily distinguished by the above characteristics.

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THE CHEMICAL AND EXPERIMENTAL EMBRYOLOGY OF *LIMNAEA*

BY CHR. P. RAVEN
Zoological Laboratory, University of Utrecht, Netherlands

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I. INTRODUCTION

A series of investigations on the embryology of *Limnaea stagnalis* L. has been made at the Zoological Laboratory of the University of Utrecht since the year 1940. The investigations were undertaken in the hope that they might throw some light on the development of those forms, the eggs of which show a precocious determination, having the so-called 'mosaic type' of development. Whereas considerable progress has been made in recent years in the study of the determination processes of eggs of the 'regulation type' (echinoderms, amphibians), the extension of our knowledge of eggs with precocious determination has not kept abreast with this. It has long been recognized that no essential difference exists between so-called 'mosaic' and 'regulation' eggs, the diversity of their modes of development being due chiefly to differences in the time at which the parts are determined in their ultimate destiny. Still, it is to be expected that the nature and course of the determination processes in the uncleaved egg or at early cleavage in 'mosaic' eggs will differ considerably from those in 'regulation' eggs, where the principal steps in the determination of organs are taken at a much later stage, when the composition of the embryo has attained a far greater degree of complexity.

Before the experimental analysis of development could be undertaken, it was necessary to make an extensive study of normal development. In particular, the 'chemical embryology', i.e. the changes in chemical composition of the embryo during development, have been studied by cytochemical methods. In this study the phase of development between the origin of the egg cells in the gonad of the adult and the formation of an embryo corresponding to the 'trochophore stage' of forms with free larvae has been taken into account.

II. METHODS

L. stagnalis is very common in ditches in the neighbourhood of Utrecht. In spring large numbers of these snails can be collected easily. For our purpose it is sufficient to bring them together in large glass aquaria, feeding them on lettuce. Daily removal of excrement and renewal of water is necessary. As a matter of fact, this method of keeping the snails is not ideal, and mortality is rather high. Noland & Carriker (1946) have recently given a description of improved culture methods for *Limnaea*.

The snails kept in the above-mentioned way only produce few egg masses. In order to obtain eggs it is, however, sufficient to add to one of the aquaria, in which the snails have been fed copiously for some days, some leaves of the floating water plant *Hydrocharis morsus ranae*, after removal of the lettuce. After 2–3 hr. (according to the temperature) the first egg masses are laid, preferably on the underside of the *Hydrocharis* leaves, and in the course of a few hours a large part of the snails present produce an egg mass.

This peculiar reaction of the snails to the addition of *Hydrocharis* (the same results have been obtained with the tropical plant *Trianea bogotensis*, which has floating leaves of approximately the same size but of a totally different structure) has been studied by L. M. van Nieuwenhoven S. J. & J. Lever (unpublished). It was found that the reaction, both as regards the total number of egg masses and the duration of the latent period, depends on the temperature. At 17–18° C. the first egg masses appeared after about 3 hr., and 50% of the snails produced an egg mass within 5 hr.; at 21–26° C. oviposition began at about 2 hr. and 70% of the snails reacted. No reaction occurred if the snails were separated from the *Hydrocharis* by a partition of gauze. Dried *Hydrocharis*, extracts of grated plants, as well as chopped or shredded leaves, had no effect, or merely a slight reaction occurred. Only the leaf-disks produce the egg-laying stimulus, the stalks scarcely having any effect. When the plants are kept submerged by weighting them with lead, a reaction ensues. The snails did not react to paraffined pieces of paper in the shape of *Hydrocharis* leaves.

A decisive step forward was made when Lever proceeded to study the influence of illumination. In the dark the snails did not react to *Hydrocharis* which had been kept in the dark for a few days previously; if, however, the plants had been illuminated strongly a short time before, a reaction occurred. When the snails were transferred to water that had been rendered rich in oxygen beforehand by passing the pure gas through it, a strong reaction occurred; on the contrary, a gradual increase in oxygen content of the water in which the snails are kept has no influence; neither had brief exposure of the snails to pure oxygen any effect. Apparently the action of

Hydrocharis can be explained in part by the oxygen produced. However, that this is not the only factor is evident from the fact that the addition of *Elodea*, which is known to be a powerful producer of oxygen, gives no reaction, even with extra illumination.

Besides the oxygen content, temperature also has an influence. A considerable but gradual rise in temperature from 18 to 28° gives a strong reaction. On the contrary, neither a gradual slight rise nor a sudden rise in temperature produced any effect.

The processes going on in the snail during the latent period of the egg-laying stimulus have been studied by Bretschneider (1948*b*). He sectioned 155 specimens of *Limnaea* before and during the first hours after adding *Hydrocharis*, and the position of the eggs was determined. Before the addition of *Hydrocharis*, in all the snails the oocytes were still lying in the gonad. At room temperature of about 17° C. the first egg passes into the oviduct after 30 min.; then, the eggs can be seen slipping one by one through the spermoviduct into the oviduct, where each egg is surrounded by its capsule membrane. After 60 min. the formation of the egg mass has begun in the pars nidamentaria of the oviduct, where the egg capsules are embedded in the common jelly, and at 2 hr. the front part of the egg mass has arrived in the pars vaginalis; the completion of the egg mass takes another hour.

III. NORMAL DEVELOPMENT

1. *Oogenesis*

The oogenesis of *Limnaea* has been studied by Bretschneider (1948). All the developmental stages of the oocyte can be found side by side in the hermaphrodite gonad all the year round. The duration of the various phases could be determined for the first generation of eggs by examining snails bred from the egg.

The gonad primordium with its primary sex cells becomes visible in a 15-day-old embryo; after another 15 days the stage of secondary oogonia has been reached. This entire first phase is characterized by intense multiplication. The young oocyte, developing from the secondary oogonium, travels in the acini of the gonad for 6 days, retro-epithelially, showing amoeboid movement. Afterwards it becomes sessile and surrounds itself with follicular cells, after which actual growth and differentiation commence, yielding a full-grown oocyte in 25 days' time. The phenomena of growth and differentiation described below take place mainly in this phase. When the oocyte has attained its definite size a phase of rest sets in, in which the oocyte is ready for fertilization; this phase can last a few days in summer but months in winter. Ovulation takes place through the autolysis of both the follicle epithelium and the acinus epithelium covering it, whereby the oocyte passes into the lumen of the gonad. Calculation of the volumes shows the egg volume to increase from $1700\,\mu^3$ to, on an average, $910{,}000\,\mu^3$ in the growth phase, i.e. it becomes 535 times as large.

During the growth of the ovum all visible changes in the generative components of the nucleus are absent. The well-developed chromosomes of the oogonium nucleus become invisible at the formation of the amoeboid oocyte, and remain so until the insemination stage. On the other hand, the vegetative function of the nucleus takes a prominent place during the growth of the egg. The nucleus plays an important part as a producer of various paraplasmatic substances.

Determination of the nucleoplasmic ratio shows that the growth of the nucleus lags behind in comparison with the growth of the cytoplasm in the amoeboid stage of development, but that from the time of follicle formation until the disposition phase, nucleus and cytoplasm keep pace with each other. Only at the end of the oogenesis does the nucleoplasmic ratio decrease further.

During the growth phase the egg exhibits a periodic shrinking and swelling of the nucleus. This pulsation expresses itself morphologically by the fact that the nucleus temporarily deviates from its spherical shape and usually displays nipple-shaped evaginations on one side. Accumulations of nucleoplasm occur in the nuclear evaginations, whilst the adjoining cytoplasm has a vacuolar character and contains no granula; this suggests that an interchange of matter between nucleus and cytoplasm is taking place here. These pulsations are attended by changes in the surface tension of the nucleus. In centrifuged ova, in some cases, the nucleus retains its spherical shape, whereas in other cases the heavy nucleolus is situated in a nuclear hernia of considerable size.

The synthetic activity of the nucleus manifests itself most strikingly in the nucleoli. The youngest oocyte has two nucleoli of different sizes, of which only the larger one remains in subsequent stages of development. In the beginning its size increases in proportion to the growth of nucleus and cytoplasm, until it has attained a diameter of 20 μ; thereafter it diminishes in size. However, great fluctuations occur, indicating an intense metabolic activity of the nucleolus. At the beginning of development of the oocyte, its nucleolus is acidophil; at the end, it is uniformly basophil; in the intermediate phases it consists of acidophil and basophil parts. Substances are produced either in the interior or on the surface of the nucleolus which are extruded into the karyolymph and pass through the nuclear membrane into the cytoplasm as granules or droplets.

The young oocyte is characterized by particularly dense cytoplasm of a basophil nature. After the formation of proteid and fatty yolk, a highly hyaline cytoplasm at the periphery remains free from inclusions. At the apex of the cell, cytoplasm collects during development, sinks down to the nucleus and surrounds this as perinuclear plasm.

The main mass of paraplasmatic reserve substances is formed by the proteid yolk, which occupies about 50% of the egg volume in newly laid eggs subjected to centrifugation. It consists of two kinds of protein granules, which may be distinguished as beta and gamma granules. Both are formed by the Golgi bodies.

In the youngest oocytes the Golgi field is situated on one side of the nucleus; it consists of a basophil substratum in which osmiophil granules are found. These granules give rise to the Golgi bodies or dictyosomes, which form the beta granules. At first they multiply by constriction; soon they get a light coloured centre, the primary germ of the future beta granule which quickly increases in size. When a size of about 4μ is reached, growth comes to an end and the dictyosome becomes smaller (to about 1μ) through condensation of the proteid substance. As the dictyosomes grow older their osmiophil properties decline and finally disappear altogether. The beta granules give the usual proteid reactions.

The gamma granules are distinguished from the beta granules by their size of 4μ, their frequently oblong shape and strongly acidophil staining. These too are formed in connexion with dictyosomes, already showing an oblong shape at an early stage.

The young oocytes possess granular mitochondria; as they grow older, more and more filiform mitochondria appear. On their surface, granular swellings are formed, which at last convert the mitochondrium into a chain of granules. In this stage the mitochondria break up, and the granules thus formed are liberated in the cytoplasm. These alpha granules show the staining properties of the mitochondria; in the fully grown oocyte their specific gravity lies between that of the cytoplasm and the proteid yolk.

Fats occur in the *Limnaea* egg as separate vacuoles, and are not, as is the case in some other ova, a component of the yolk granules. The fat is formed without visible cell structures, mostly peripherally in the cytoplasm, in the form of vacuoles which often combine to form large drops. The first fat is formed at the end of the amoeboid phase; from this time on it gradually increases until it occupies about 5% of the volume of the egg at the end of oogenesis. It is more or less evenly distributed throughout the whole egg. Besides neutral fats, lipoids can be demonstrated in the egg.

Glycogen is not formed by visible cell structures, but is, as a rule, present in a diffuse form, rarely granular. In the latter case it appears that the glycogen at first collects, in a weak concentration, in vacuoles; through condensation these become granules. The glycogen occurs during follicle formation together with the fat and shows a slight increase in the first half of oogenesis and a greater in the second.

During the whole of oogenesis the Feulgen reaction is negative. Only in old oogonia and young oocytes do the chromosomes contain thymonucleic acid; later on it is only present after ovulation, when the tetrads are formed.

In young oocytes the cytoplasm contains a high concentration of ribonucleic acid; the content declines during development, possibly in proportion to its being consumed for building up the paraplasmatic inclusions. It appears chiefly in the periphery of the cell, in centrifuged ova in the hyaloplasm layer. The beta and gamma granules contain no ribonucleic acid at first; towards the end of oogenesis it seems to be absorbed by the beta granules, since these show a high content of this acid after oviposition. Within the nucleus the ribonucleic acid is localized exclusively in the basophil parts of the nucleolus and its derivatives.

Glutathione is first formed in the nucleolus, which contains this substance in all stages, both in the acidophil and basophil parts. An emission of glutathione from the nucleolus into the nucleoplasm was frequently observed. The cytoplasm also contains glutathione, and its content diminishes during oogenesis. When the nucleus dissolves in the fertilization phase, the nuclear glutathione is released and distributes itself diffusely, showing a higher concentration chiefly around the maturation spindle. Apparently the glutathione exists in the reduced form at all stages.

Indophenol blue oxidase occurs in the shape of fine granules, which, in a young oocyte, lie as a little group in the neighbourhood of the dissolving Golgi field. During intrafollicular growth the oxidase is accumulated in a broad field in the region of the nucleus. The follicular cells are also distinguished by a high content of this enzyme. In fully grown oocytes the granules lie together in groups of two to five throughout the whole periphery of the egg.

Vitamin C is present only in small quantities, in the shape of little granules lying in the periphery of the dictyosomes.

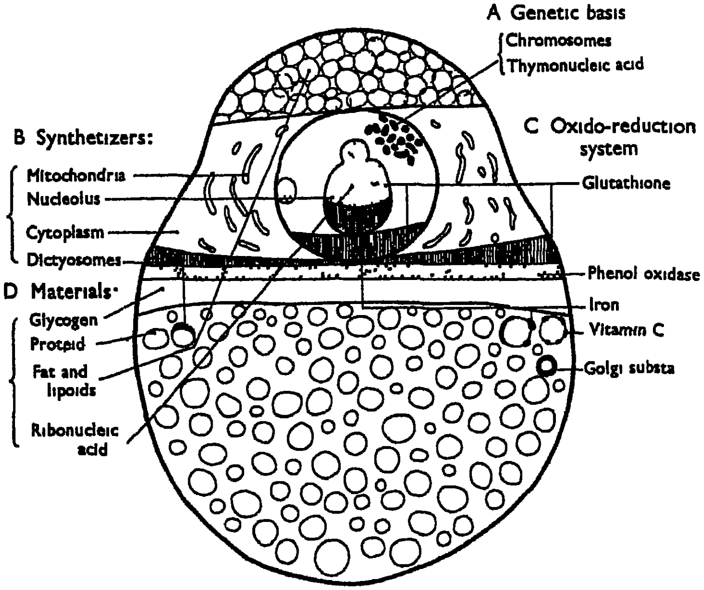

Fig. 1. Diagram summarizing the structure of the fully grown oocyte of *L. stagnalis*; the substances have been separated as much as possible by centrifugation.

During yolk formation a yellow pigment belonging to the group of melanines appears; it is bound to the beta granules. Each granule contains this pigment bound to the proteid in a diffuse form.

Non-masked iron appears after the formation of the follicle, diffusely distributed throughout the perinuclear plasm. A broad peripheral strip of cytoplasm always remains free from iron. Afterwards iron is found localized in most oocytes in coarse granules in the cytoplasm and in the dictyosomes; the distribution of the iron corresponds to that of the osmiophil Golgi substance. As the beta and gamma granules grow older they contain appreciably less iron; in the newly laid eggs, however, a great deal of iron occurs, bound to the beta granules. The nucleolus also contains iron, as a rule only in the basophil parts; the products of the nucleolus likewise contain iron, but they do not remain for long as such in the nucleus.

Fig. 1 summarizes the structure of the fully grown oocyte of *L. stagnalis*, as it presents itself after centrifugation which leads to the separation of the various substances of the egg.

2. *Insemination*[*]

The growth phase of the egg is followed by a rest phase of variable length, in which the spherical oocyte is situated within the follicle at the wall of the gonad, with the animal pole pointing inwards. At ovulation the egg comes into the lumen of the gonad, from which it is transported to the spermoviduct. At this moment the shape of the egg becomes irregular and elongated; presumably the tension at the surface is greatly reduced. In the spermoviduct the eggs meet the spermatozoa originating from the copulation partner, and insemination takes place.

The spermatozoa leave the gonad by the efferent ducts and collect in the spermoviduct, where they chiefly fill the pouch-shaped evaginations of the latter (vesiculae seminales) (Fig. 2). From the spermoviduct the spermatozoa make their way through the narrow vas afferens and reach the glandula prostata, where mucus and albumen are copiously exuded. At copulation the sperm passes through the vas efferens and the penis, thus reaching the pars vaginalis of the partner, where it is stored for the time being in the receptaculum seminis. The wall of this organ secretes an orange-yellow pigment and proteids which mingle with the sperm. Small portions of sperm are repeatedly passed on to the vagina. These balls of sperm travel along a ciliated channel running upwards along the entire length of the oviduct, through which the sperm is passed by ciliary movement, until they reach the spermoviduct. Here the spermatozoa release themselves from the sperm substance, perhaps with the aid of a cadmium-yellow secretion produced by the cells of the oviduct.

When the eggs have entered the spermoviduct, the wall of the oocyte nucleus shrinks away, and sixteen tetrads become visible. The cytocentrum of the egg divides itself, forming the two poles of a spindle, in which the tetrads arrange themselves. Simultaneously, the spermatozoa force their way into the oocyte. As a rule polyspermy occurs. At the spot where a spermatozoon has entered, a basophil substance is formed, probably arising from large basophil granules in the cytoplasm, which closes the cell membrane by forming a coagulation plug. The whole spermatozoon, including the middle part and the tail, enters the oocyte and often makes its way through the whole egg, the convoluted tail being seen for considerable parts of its length in the cytoplasm. Finally, the head of the spermatozoon comes to a halt in the egg cortex and breaks away from the tail. Only one of the sperm nuclei maintains itself, the superfluous spermatozoa melt away and are dissolved.

3. *Mechanism of oviposition*[*]

On their way through the oviduct the fertilized ova are enclosed by several layers and united into an egg mass. At short intervals an egg slides from the spermoviduct into the first part of the oviduct (pars ventricularis) with a jerking movement.

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[*] Bretschneider (1948*b*).

A sucking action is set up by dilation of the thick muscular wall of this part, whereby at the same time a stream of secretion is sucked from the glandula albuminifera. The pars ventricularis swells to approximately the size of a future egg capsule; the oocyte is, therefore, surrounded by as much perivitelline fluid as is necessary for forming an egg capsule; this fluid chiefly contains proteins and salts.

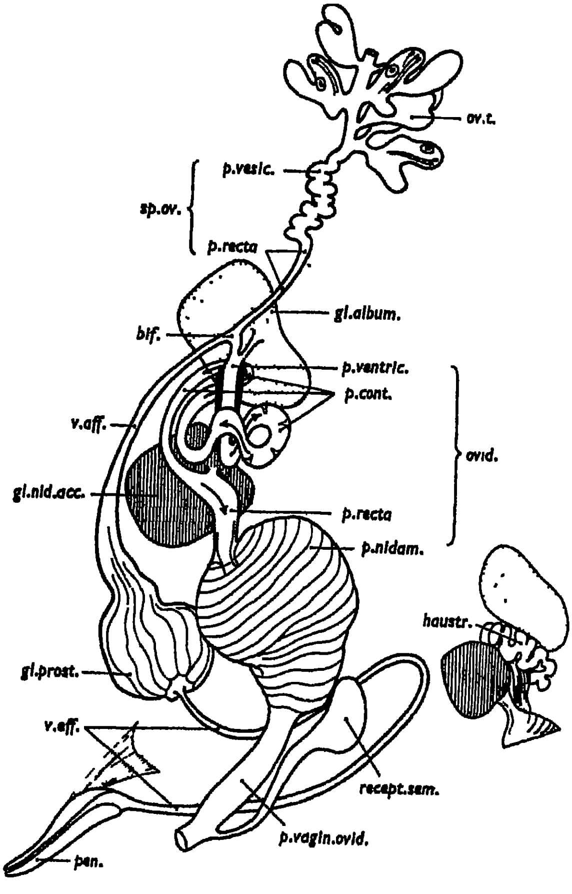

Fig. 2. Genital tract of *L. stagnalis* (somewhat schematic). *bif.*, bifurcatio spermoviducti; *gl.album.*, glandula albuminifera; *gl.nid.acc.*, glandula nidamentaria accessoria; *gl.prost.*, glandula prostata; *haustr.*, haustra of the pars contorta oviducti; *ov.t.*, ovariotestis; *p.ventric.*, *p.cont.*, *p.recta*, *p.nidam.*, *p.vagin.ovid.*, pars ventricularis, pars contorta, pars recta, pars nidamentaria, pars vaginalis oviducti; *pen.*, penis; *recept.sem.*, receptaculum seminis; *sp.ov.*, *p.vesic.*, *p.recta*, pars vesicularis, pars recta of the spermoviduct; *v.aff.*, vas afferens; *v.eff.*, vas efferens.

Through contraction of the muscular wall, ovum and perivitelline fluid are forced into the following part of the oviduct (pars contorta) where the egg capsule membrane is formed. The pars contorta exhibits haustra-like enlargements; directly in the first haustrum, which is of the size of an egg capsule, a thin tough layer is formed on the surface of the perivitelline fluid, the inner capsule membrane. When the egg capsule moves farther through the pars contorta, a thin layer of mucus is poured around it in each succeeding haustrum, whereby the stratified outer capsule membrane is formed.

In the following part of the oviduct, pars recta, the successive egg capsules are cemented together by a mucous secretion from the glandula nidamentaria, which forms the tunica interna of the egg mass. Next, the egg capsules pass through a sort of valve and enter the wide pars nidamentaria. By means of a rhythmic secretion of the walls of this organ new layers of mucus are deposited around the tunica interna, which forms the tunica capsulae, the outer layer of the egg mass. Finally, this mass passes through the pars vaginalis and issues from the genital orifice.

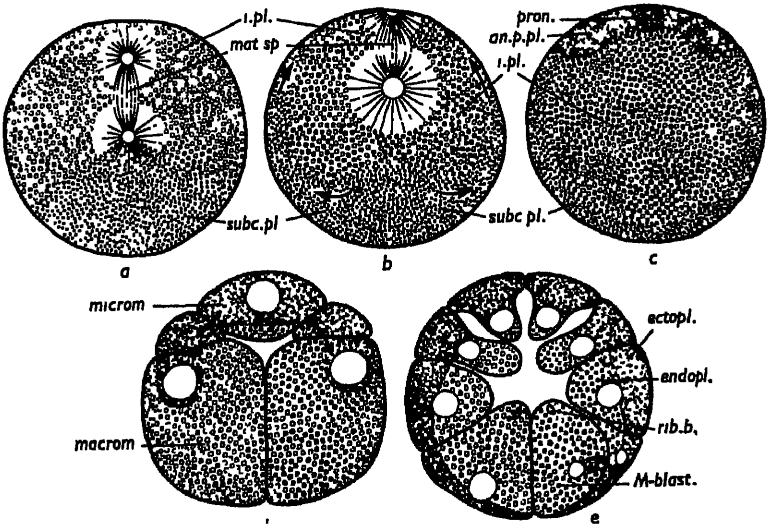

Fig. 3. Chemodifferentiation in the egg of *L. stagnalis*. *a*, newly laid egg, *b*, extension of the subcortical plasm (*subc.pl.*) in animal direction; *c*, formation of the animal pole plasm (*an.p.pl.*); *d*, 8-cell stage, accumulation of denser plasm in the micromeres; *e*, distribution of ectoplasm (*ectopl.*) and endoplasm (*endopl.*) over the cleavage cells. *i.pl.*, inner plasm; *macrom.*, macromeres; *microm.*, micromeres; *mat.sp.*, maturation spindle; *M-blast.*, primary mesomere; *pron.*, pronuclei; *rib.b.*, dark bodies containing much ribonucleic acid.

4. *Development of the fertilized egg**

The uncleaved stage of the egg lasts 4–5 hr. at a temperature of about 20° C. The first polar body is formed, on an average, 60–70 min. after laying, the second polar body about 1 hr. after the first. The egg exhibits amoeboid movements shortly after each of the two polar bodies has been formed. The first cleavage division occurs about 120–130 min. after the formation of the second polar body.

The egg is surrounded by a vitelline membrane. This membrane evidently undergoes a change in consistency during the uncleaved stage, which appears from the fact that the first polar body is pressed through it, whereas the second polar body partly pushes the membrane before it, and thus comes to lie inside the membrane.

* Raven (1945, 1946*a*).

In the newly laid egg a special substance is found at the vegetative side (Fig. 3*a*). It is the vegetative pole plasm, which is stained blue by azan after fixation in Bouin's fixative; it contains a dense mass of protein granules of moderate size, the beta granules. The rest of the egg consists of plasm which is orange in azan-stained sections; it contains a few scattered blue beta granules and many larger protein granules (gamma granules) of a deep red colour, furthermore a great many fine orange-red mitochondria (alpha granules). The difference between the two kinds of plasm is not only due to their different content of granular inclusions, but concerns the intergranular hyaloplasm as well. The rays of the asters of the maturation spindle always correspond in colour to the plasm in which they are lying, which is especially evident when one of the asters is situated exactly on the borderline of the two kinds of plasm.

A short time after oviposition, the vegetative pole plasm begins to extend under the surface of the egg in the direction of the animal pole. At first it leaves free a *circular area at the animal pole (Fig. 3b), in which the polar bodies are formed.* After the extrusion of the second polar body this opening is closed and the blue stained plasm now surrounds the whole egg; we shall call it, henceforth, the subcortical plasm. In the orange-red inner plasm, which is now quite enclosed by it, vacuoles are formed by the swelling of the gamma granules. About 1 hr. before the first cleavage a very dense plasm collects on the animal side of the egg: the animal pole plasm (Fig. 3*c*). It contains many alpha granules and stains a dark violet with iron haematoxylin. Apparently, this plasm arises from an accumulation of substances which are originally distributed more or less evenly throughout the egg.

The cleavage of *Limnaea* is characterized by the very early occurrence of a cleavage cavity. Already at the 2-cell stage a wide cavity is formed between the two blastomeres, and this is repeated in the succeeding cleavages. The cleavage furrows at first cut very deeply into the egg, so that finally the daughter cells touch each other at one point only. The egg cortex and subcortical plasm penetrate into the egg together with the cleavage furrow (Fig. 4*a*). When afterwards the daughter cells flatten themselves against each other, the cortex forms the dividing wall between the two blastomeres (Fig. 4*b*). In this dividing wall slit-like cavities are soon formed, which fuse to form the cleavage cavity. The latter is narrow at first; on the adjoining parts of the blastomeres, however, hyaline 'secretion cones' (Fig. 4*c*) are formed, which secrete a great deal of fluid into the cleavage cavity, whereby this soon widens (Fig. 4*d*). When the cleavage cavity has attained a certain size, it breaks through to the outside and the fluid is ejected, as described by Comandon & de Fonbrune (1935). This process may be repeated several times between cleavages.

In the first two cleavages the substances of the egg are distributed passively among the blastomeres without any appreciable shifting of material taking place. Immediately preceding the third cleavage, however, the subcortical plasm concentrates on the animal side and fuses here with the animal pole plasm, as well as with the dense plasm surrounding the nuclei, to form one single mass; the vacuolar inner plasm is pushed away to the vegetative side. In this way, the latter passes chiefly into the macromeres, whilst the micromeres consist almost entirely of dense plasm and possess only a narrow border of vacuolar plasm on the side nearest the cleavage cavity (Fig. 3*d*). The dense plasm contains many mitochondria and beta granules, furthermore much ribonucleic acid and sulphydril compounds, whilst the vacuolar plasm chiefly contains swollen gamma granules and droplets of fat.

In the succeeding cleavages this differential division of the two plasm substances is carried still further. At each division the two daughter cells each get a part of both substances, but the animal cell gets more of the dense, the vegetative cell more of the vacuolar plasm. The dense plasm comes to lie in all the cells on the side facing outward, and forms the ectoplasm; the vacuolar plasm forms the endoplasm, which fills the part of the cell facing the cleavage cavity. In consequence of the unequal distribution of the two substances, the relative quantity of ectoplasm finally decreases from the animal to the vegetative side, that of the endoplasm in the opposite direction (Fig. 3*e*).

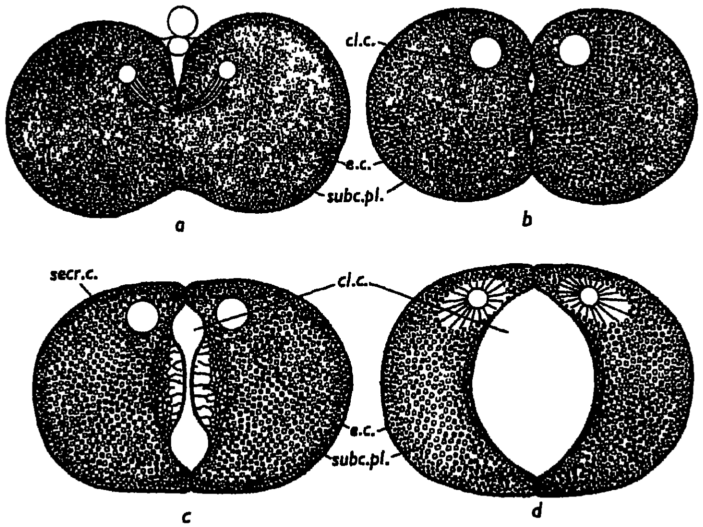

Fig. 4. The first cleavage and the formation of the cleavage cavity in *L. stagnalis*. *a*, beginning of cleavage furrow; *b*, formation of the cell wall, beginning of formation of cleavage cavity (*cl.c.*); *c*, widening of cleavage cavity through action of secretion cones (*secr.c.*); *d*, wide cleavage cavity. *e.c.*, egg cortex; *subc.pl.*, subcortical plasm.

The mesomeres are formed by a division slanting inwards; consequently, the mesoderm cells contain very little ectoplasm or none at all (Fig. 3*e*). Furthermore, the cells of the fourth quartet are distinguished by having characteristic little bodies in the central part of the cell, which have a high content of ribonucleic acid (Fig. 3*e*); in later stages these apparently dissolve in the protoplasm. The three endomeres of this quartet contain more glycogen than the other cells. Finally, mention should be made of the formation of peculiar thickened rings on the outer surface of four equatorial groups of cells, probably belonging to the second quartet; the meaning of these rings is not yet clear.

The composition of the yolk undergoes a considerable change in later cleavage stages. The swelling of the gamma granules decreases. The number of these granules increases, whilst at the same time the beta granules disappear. Meanwhile, the cells absorb albumen from the surrounding egg capsule fluid; this is stored in the form of albumen vacuoles in the ectoplasmic part of the cells. The nuclei probably play a part in this transformation of the yolk. They are situated in all the cells on the boundary line of ectoplasm and endoplasm (Fig. 3*e*). The nucleoli, which have a high content of ribonucleic acid and sulphydril compounds, show great activity. Intra-nucleolar vacuole formation, with expulsion of the product into the karyolymph, occurs very generally, whilst ejection of whole nucleoli into the cytoplasm is not uncommon. The cytoplasm surrounding the nuclei contains much glutathione. Furthermore, the Golgi bodies of the cells seem to take part in the transformation of the yolk.

At gastrulation a change in the shape of the cells at the vegetative pole causes the invagination of the archenteron. The cells of the vegetative side, which are poor in ectoplasm, become the endoderm, whereas the cells of the animal side, having a large proportion of ectoplasm, form the ectoderm; finally, the mesoderm arises from cells which are nearly or entirely devoid of ectoplasm. This suggests that the distribution of substances brought about by the 'chemo-differentiation' of the egg affords a sufficient explanation for the separation of the germ layers; it has, however, not yet been possible to put this hypothesis to the test.

At the gastrula stage the albumen vacuoles in the ectoderm become smaller with the exception of a few cells situated at the animal pole, where these vacuoles swell to enormous proportions. Furthermore, this stage is distinguished by a considerable increase in the thymonucleic acid content of the nuclei. Whereas in younger stages thymonucleic acid could be detected in the chromosomes during mitosis only, from the gastrula stage onward the resting nuclei also contain a considerable quantity of this acid.

Towards the end of the gastrulation period the differentiation begins, first in the ectoderm, then also in the other germ layers. In the ectoderm two kinds of cells may be distinguished: large flattened ciliated cells occupy the region of the head vesicle, apical plate and velum, whilst the remaining ectoderm consists of small prismatic cells. In comparison with the small-celled ectoderm, the large ciliated cells contain more and larger albumen vacuoles and have a higher content of iron (Arendsen de Wolff-Exalto, 1947) and glycogen, of Golgi substance and mitochondria, of peroxidases and phenolases. On the other hand, their cytoplasm contains no ribonucleic acid and the nuclei contain little thymonucleic acid. The cytoplasm of these cells consists of three zones (Fig. 5): the outer zone contains a large quantity of diffusely distributed lipoids, the middle zone of albumen vacuoles and the basal layer of yolk granules are both distinguished by containing many Golgi systems, mitochondria and very much glycogen. The albumen vacuoles of the middle zone, contents of which show an alkaline reaction, absorb and store up neutral red and nile blue hydrochloride from diluted solutions.

Between the large ciliary cells of different regions minor differences exist. Special mention should be made of two pairs of cells, at the transition of the velum and the head vesicle, whose nucleoles differ from those of all the other cells in respect of their staining properties, and which furthermore contain large quantities of peroxidases.

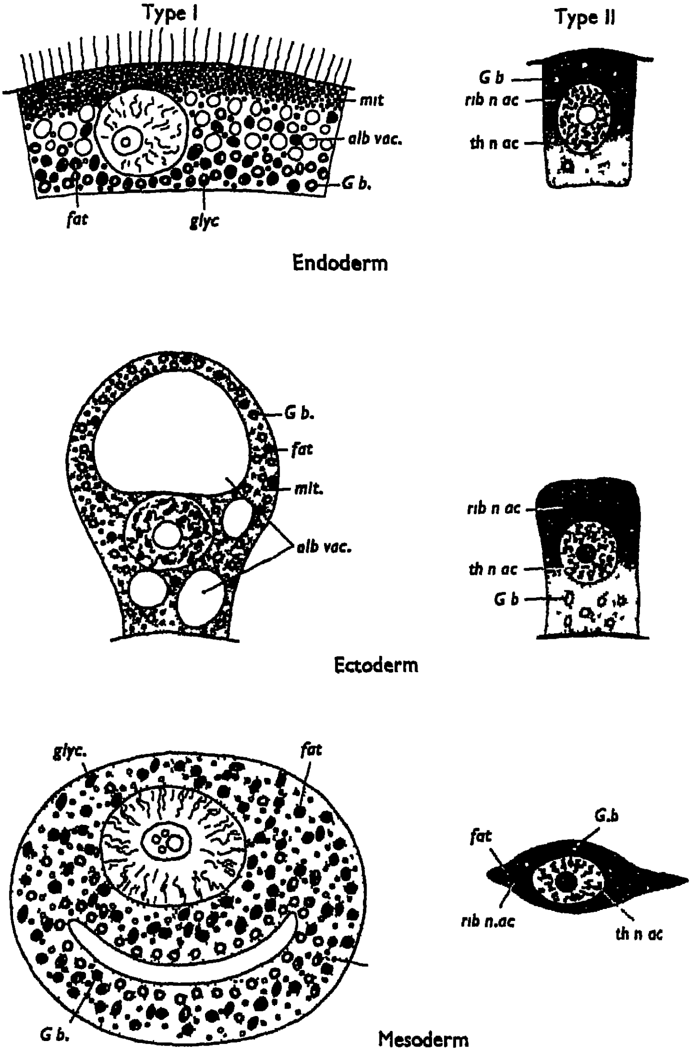

Fig. 5. Chemical differentiation in the ectoderm, endoderm and mesoderm of *L. stagnalis*. *alb.vac.*, albumen vacuoles; *G.b.*, Golgi-bodies; *glyc.*, glycogen; *mit.*, mitochondria; *rib.n.ac.*, ribonucleic acid; *th.n.ac.*, thymonucleic acid. Types I, II, cf. text.

The ciliated cells, which develop later in the dorsal wall of the mouth cavity and the ventral wall of the oesophagus, resemble those of ectoderm.

The small prismatic ectoderm cells have nuclei containing much thymonucleic acid, but having a nucleolus without ribonucleic acid; the cytoplasm contains much ribonucleic acid and glutathione, but little Golgi substance, fat, glycogen and iron, and few mitochondria; the albumen vacuoles finally disappear altogether. The shell gland is distinguished by a great concentration of phenolases and peroxidases; the localization of the two enzymes is still somewhat different, as the peroxidases have their greatest concentration in the periphery of the shell gland region, the future mantle fold, whereas the phenolases are most highly concentrated in the shell itself.

The cells of the mouth cavity and radular sac resemble those of the small-celled ectoderm very much, but the plasm contains somewhat more iron, especially on the side facing the lumen; the oesophagus, on the other hand, is composed of cells with highly vacuolated protoplasm containing very little ribonucleic acid.

In the endoderm the cells differentiate in two directions: on the one hand, small gut epithelium cells are formed, on the other hand large albumen cells (Fig. 5). In the former the albumen vacuoles gradually disappear, in the latter they become enormously enlarged. The albumen in these vacuoles shows an acid reaction; the albumen is digested both intracellularly and, with the aid of drops of secretion, extracellularly in the gut. The small endoderm cells contain much ribonucleic acid and bound sulphydril compounds. The albumen cells have much less of these substances, but are rich in mitochondria and fat drops, and contain more iron than the small-celled endoderm.

In the mesoderm it is chiefly the cells of the protonephridia which distinguish themselves from the other, mesenchyme forming, cells (Fig. 5). The latter have nuclei with a moderate quantity of thymonucleic acid; the cytoplasm is rather rich in fat and osmiophilic lipoids, and contains much ribonucleic acid. In later stages special elements lying subepidermally are formed, very rich in phenolases, and others containing large quantities of peroxidases. The cells of the protonephridia, especially the giant cells, are distinguished by possessing nuclei which at first contain much and afterwards little thymonucleic acid and of which the nucleoli show great activity, a large number of intranucleolar vacuoles being formed, and contain no ribonucleic acid. The cytoplasm is osmiophil and contains many mitochondria and fat drops, a great deal of glycogen and iron, but only little ribonucleic acid.

Summarizing, one can say that in all three germ layers the chemical differentiation leads to a separation into two types of cells, which specialize in different directions (Fig. 5).

It is reasonable to suppose that these two types of cells represent metabolic types. The larval organs already functioning (cilia, albumen-digesting cells and protonephridium) contain much fuel (fat, glycogen) for their working metabolism, and have an active system of Golgi-mitochondria; cell type II, which serves for further building up the body, contains especially the building materials thymo- and ribonucleic acid. In this way the morphogenetic significance of the cells is expressed in their composition and metabolism.

IV. EXPERIMENTAL ANALYSIS OF DEVELOPMENT

1. *Osmotic properties of the egg*

The eggs of *L. stagnalis* are generally of a somewhat irregular shape immediately after being laid. In a few minutes they become rounded, assuming the shape of an ellipsoid, the main axis of the egg being slightly shorter than the transversal diameter. The sizes of the eggs of different batches vary a good deal; the eggs of one and the same batch, however, agree with one another fairly accurately. The average dimensions are about $117 \times 122\mu$, which implies an average volume of $912{,}000\mu^3$.

During the time elapsing until the beginning of the first cleavage, the eggs increase considerably in size; the volume increases by approximately 35–55%. This swelling of the egg must be caused by absorption of substances, especially water, from the surrounding egg capsule fluid. In connexion with this, the osmotic properties of the egg have been studied (Raven & Klomp, 1946).

Eggs removed from their capsules and placed in distilled water immediately after laying show a high degree of swelling. In one of the experiments a swelling of 105% in 3 hr. time was found. Nevertheless, the first two cleavages can take place, though not in quite a normal way. However, the development does not advance further than the 4-cell stage, not even when the eggs are placed is distilled water after the first, or even after the second, cleavage. In this stage the embryo is evidently susceptible to hypotonicity.

Further experiments were made with solutions of urea and sucrose of varying concentrations, in which the eggs were placed immediately after being laid. It appeared that the molar concentration of the solution determined the behaviour of the eggs; in solutions stronger than 0·10 M the eggs shrink, in those weaker than 0·09 M swelling occurred. Evidently, we are here dealing with a purely osmotic phenomenon; furthermore, we may infer from the results that the eggs are, on an average, isotonic with a 0·093 M solution of non-electrolytes immediately after laying.

A few other facts may also be deduced from the experiments. From the equilibrium volumes attained in solutions of different molar concentrations, the 'non-solvent volume' was calculated; on an average, it amounts to 57% of the original volume of the eggs. This calculated volume is extraordinarily high; the low elasticity of the vitelline membrane, which resists expansion, presumably plays a part here. The permeability constant for swelling in distilled water was calculated; expressed in the units of Brooks & Brooks (1941), it amounted to about $1\text{–}2 \times 10^{-7}$, a value which agrees perfectly well in order of magnitude with results obtained for many marine eggs.

Eggs placed in a 0·15 M solution of urea directly after oviposition, still extrude the two polar bodies, but do not cleave. In weaker solutions of urea (0·03–0·10 M) nearly all the eggs attain the 4-cell stage; they do not develop further, however, but soon show cytolysis. As in the experiments with distilled water, a specific sensitivity of the eggs in the 4-cell stage appears from this. That the osmotic pressure of the solutions alone does not play a part in this case, but that the chemical properties are also of importance, is proved by the fact that quite different results are obtained with sucrose. Here also, to be sure, no cleavage occurs in 0·15 M and stronger solutions, but in a slightly weaker concentration of the sugar solution cleavage takes place, in which the stage of development attained is further advanced according as the concentration of the solution is lower. In solutions of about 0·07–0·08 M development advances furthest; in these a stage of about twenty-five to thirty blastomeres can be reached. If the concentration is made still lower the stage of development attained becomes less advanced again, until a final stage of four cells is reached in pure distilled water.

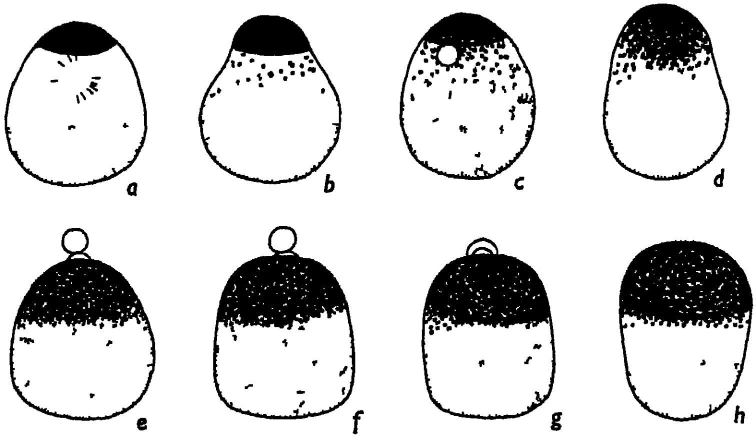

Fig 6 Eggs of *L stagnalis* centrifuged (5 min, 3800 r p m) at different moments during the uncleaved stage The first was centrifuged half an hour before first polar body formation, the others successively later with intervals of, on an average, about 30 min ; the last 10 min. before the beginning of cleavage.

2. *Centrifugation experiments*

When eggs of *Limnaea* are centrifuged a distinct stratification is obtained. Fig. 6 shows a series of eggs which have been centrifuged in their capsules by means of an electric centrifuge during 5 min. at a speed of 3800 r.p.m. (centrifugal pressure: 1860 × gravity), beginning at different moments during the uncleaved stage. In general, four zones may be distinguished in these centrifuged eggs: (1) a centripetal grey cap, (2) a zone of clear protoplasm, (3) a narrow greenish layer of granules, (4) a centrifugal yellow cap. In comparing the eggs centrifuged at different moments, it becomes evident that the relative proportions of these zones show regular changes during the uncleaved stage. Zone (1) which is only small at first, greatly increases in size, whereas zone (4) shows a decrease. The absolute volumes of the zones have been calculated from photographs (Raven, 1945), with the following results (Table 1).

It is clear from this table that the great increase in the volume of the egg during the first maturation division is mainly caused by zone (4), whereas the other zones remain practically constant. Then zone (4) decreases again in size, whilst zones (1) and (2+3) increase very much in volume. Finally, the increase in the volume of the egg is almost entirely to the benefit of zone (1); zone (2+3) returns to its original volume, whereas zone (4) is reduced in size. These peculiar changes in size of the layers may be explained by the swelling and resultant decrease in density of the gamma granules, which at first are heavy and accumulate in zone (4), but collect in zone (1) after swelling. From Table 1 it appears that the increase of zone (1) by approximately 500,000 μ^3 results from a reduction of zone (4) by approximately 130,000 μ^3 and an increase in the egg volume of approximately 360,000 μ^3. From this it may perhaps be conjectured that the gamma granules have absorbed, on an average, nearly three times their own volume of water.

Table 1. *Volumes of zones in centrifuged eggs*

Stage	Zone (1)		Zones (2+3)		Zone (4)		Total volume 1000 μ^3
	1000 μ^3	%	1000 μ^3	%	1000 μ^3	%	
27 min before first polar body	58	5.2	480	43.5	567	51.3	1105
12 min after first polar body	65	4.6	516	37.1	811	58.3	1392
3 min after second polar body	111	7.7	628	43.4	707	48.9	1446
20 min before first cleavage	567	38.8	460	31.5	435	29.7	1462

The degree of stratification brought about by centrifuging the eggs at different moments with constant velocity during a fixed number of minutes can be used as a means of estimating relative viscosity changes. Viscosity is low if the eggs are fully stratified, it is relatively high if stratification is less distinct. As Fig. 6 shows, a regular series of viscosity changes during the uncleaved stage of the egg is indicated. Immediately after laying viscosity is low; it remains so during the first maturation division, begins to rise shortly before the extrusion of the second polar body, reaches a maximum about half-way between second maturation and first cleavage, then drops rapidly, has a minimum half an hour before cleavage, and rises again until cleavage (Fig. 7).

These observations have been extended by Heikens (1947) to the period including the first three cleavages of the egg. The viscosity is low immediately before each cleavage; it begins to rise when the cleavage furrow makes its appearance, attains a maximum about 10–15 min. after the beginning of cleavage, then drops gradually until the beginning of the next division (Fig. 8). In relation to the mitotic cycle, it proves to be low during prophase, metaphase and early anaphase, begins to rise during late anaphase or early telophase and is maximum during late telophase of each division.

Finally, the degree of elongation, brought about by centrifugation at different moments during the uncleaved stage, can be used for the evaluation of the 'tension at the surface' (cf. Harvey, 1931). The distortion produced by centrifugation can be expressed in the relation L/B between the diameter L in the direction of the centrifugal force and the largest diameter B perpendicular to it. The inverse quotient B/L will change in relation to the variations in 'tension at the surface', provided that the viscosity of the egg does not change. In this way it was found that important changes of this tension take place during the uncleaved stage of the egg. It is low at first polar body formation, some time after the extrusion of the second polar body and immediately before cleavage, whereas it is higher prior to maturation and in the intervals between those minima (Fig. 7).

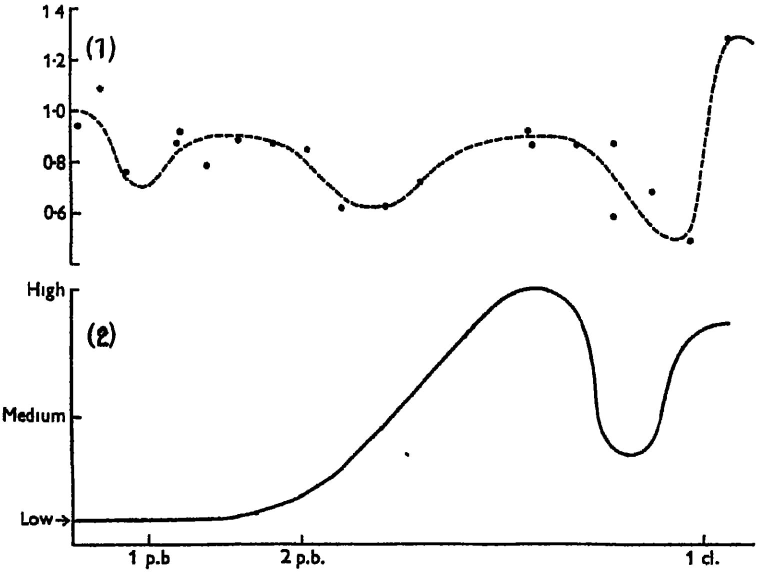

Fig. 7. Graph of the changes in 'tension at the surface' (dotted line) and in viscosity (continuous line) of *L. stagnalis* eggs from oviposition until the first cleavage. Abscissae: time. Ordinates: (1) estimated relative value of viscosity; (2) tension at the surface, expressed in the relation breadth/length of the centrifuged eggs.

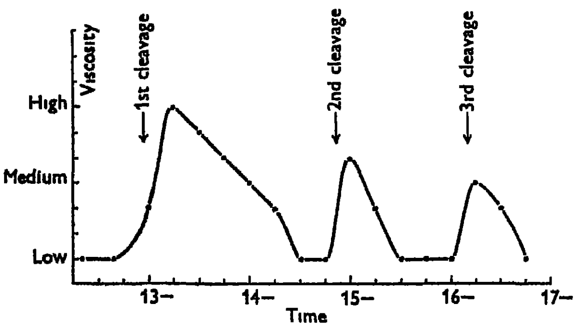

Fig. 8. Graph showing variations of viscosity during first, second and third cleavage of *L. stagnalis*. Abscissae: time. Ordinates: estimated relative value of viscosity. After Heikens (1947).

De Vries (1947) studied the influence of lithium chloride and calcium chloride solutions of various concentrations on the viscosity and 'tension at the surface' of uncleaved eggs. His experiments showed that the viscosity is lowered by 0·1–1·0 % LiCl and 0·1–1·5 % $CaCl_2$ solutions. Somewhat greater dilutions gave no effect, but with still greater dilutions (0·006–0·03% LiCl and 0·05% $CaCl_2$ solutions) a significant increase of viscosity was observed. As to the 'tension at the surface', this was increased by 0·6–1·0% LiCl and 1·5% $CaCl_2$ solutions, whereas a 0·1% LiCl solution caused a decrease of this tension.

The development of eggs centrifuged at different moments during the uncleaved stage for 5 min. at 1860 × gravity has been studied (Raven & Bretschneider, 1942). On the basis of a large number of experiments, in which altogether about 3200 eggs were centrifuged, it could be ascertained that these eggs develop into normal young snails in a large percentage of cases. On an average 68% of normal snails developed from centrifuged eggs; in the controls this was 77%. If the uncleaved stage be divided into four periods of about equal length, namely: (1) before the formation of the first polar body, (2) between the first and second polar body, (3) 0–60 min. after the second polar body, and (4) the last hour before the first cleavage, then the percentages of normal development of the eggs centrifuged in these periods were: 71, 61, 66 and 74%, respectively. So it appears that the harmful effects of centrifugation are only slight and vary little with the time of centrifugation. An increase in injurious effect toward the end of the uncleaved period certainly does not occur. This is in contradiction to the earlier statements of Conklin (1910) and Clement (1938), who, however, made too small a number of experiments.

Similar observations have been made during the 2-cell stage by Miss C. A. M. Corporaal (unpublished). As she centrifuged the eggs during 10 min. at 1130 × gravity, her results are not strictly comparable to those mentioned above. Nevertheless, it can be deduced from the experiments that centrifugation during the 2-cell stage is more harmful than at the uncleaved stage. Whereas 95% of the controls developed to young snails, in the centrifuged eggs only 45% showed a normal development till hatching. Mortality in the eggs centrifuged during the first phases of first cleavage (formation of cleavage furrow) and during the last period preceding second cleavage (eggs with wide cleavage cavity) is, on an average, somewhat higher than in the eggs centrifuged in the intermediate phases (flattening of the blastomeres against each other); however, these differences are not fully significant.

The composition of the four zones of the centrifuged eggs has been studied in sectioned material (Raven & Bretschneider, 1942). In eggs centrifuged immediately after being laid, zone (1) consists of a rather compact layer of fat. From its cytochemical reactions, it can be concluded that it contains especially unsaturated glycerides. In eggs centrifuged later, the watery vacuoles formed by the swelling of the gamma granules are added to this zone. Zone (2) consists of hyaloplasm and usually contains the maturation spindle or egg nucleus. In eggs centrifuged in later stages this zone is not distinctly separated from the vacuolar first zone. The hyaloplasm contains glutathione and ribonucleic acid. Zone (3) is a narrow band of alpha granules (mitochondria). It could be shown cytochemically that indophenol blue oxidase, benzidine peroxidase and glycogen are concentrated in this layer. Finally, zone (4) contains the beta granules and in the newly laid egg also the gamma granules; they represent the proteid yolk of the egg.

The substances of the egg displaced by centrifugation do not remain lying in their new position, but redistribute themselves throughout the egg. Protoplasmic currents during the formation of the polar bodies, and the formation and growth of maturation asters, evidently play an important part in this. In eggs centrifuged shortly after being laid this causes the stratification of the egg to disappear already before cleavage commences, so that the normal distribution of the substances of the egg is restored.

In this readjustment of the egg substances two different processes are involved: first, the dispersion of the substances accumulated during centrifuging, leading to an equal or nearly equal distribution throughout the egg protoplasm; secondly, a localization of special structures and substances, bringing about the 'chemo-differentiation' of the egg. Of the latter category of phenomena, I will mention two instances: (1) in eggs centrifuged before maturation, the polar bodies are formed at various distances from the centripetal pole; apparently, the maturation spindle returns to the original animal pole irrespective of the direction of stratification; (2) an hour before first cleavage, in centrifuged eggs the substance of the animal pole plasm collects at the animal side of the egg, as in normal eggs. We may conclude that the factors, which are responsible for the localization of substances, are not displaced by the action of centrifugal force; presumably, they are located in the relatively immovable egg cortex.

A special study has been made of eggs centrifuged shortly before the first cleavage (Raven, 1946*b*). It could be imagined that in such eggs the redistribution of substances would be interrupted by the formation of the cell walls, so that the blastomeres would remain with a permanent difference in material composition. With regard to the fat it could be easily shown, however, that also in such eggs it is redistributed throughout the egg. This is, in the first place, effected by the fact that the fat mass is drawn out along the cleavage furrows; at later stages an interchange of fat is apparently also carried on through the cell walls, so that the normal fat distribution is restored in the trochophore stage.

For the other constituents of the egg similar conditions prevailed. First of all the alpha granules begin to distribute themselves throughout the egg; 100 min. after centrifugation the layer of alpha granules has entirely disappeared. This speedy redistribution, together with the fact that the layer of alpha granules occupies approximately the middle of the egg directly after centrifugation, results in each of the first four blastomeres containing a more or less equal quantity of these granules; as in normal eggs they collect chiefly at the animal pole. It can be supposed that such conditions as exist for the alpha granules also prevail with regard to the important substances concentrated in the same layer; viz. glycogen and the enzyme granules.

In contradistinction to this, no considerable regrouping of the beta granules, accumulated on one side by centrifugation, takes place in the early cleavage stages; this brings about a very unequal distribution throughout the cleavage cells, some of which are quite full of these yolk granules, whereas others contain practically no beta granules. From the fact that, nevertheless, no serious disturbances in development occur, it may be inferred that these protein granules do not play an important part in the determination of the cells and so, in accordance with Conklin's opinion (1910), represent only a food reserve for the developing egg.

In later cleavage stages, when the transformation of the proteid yolk begins, protein granules appear also in the cells originally containing little yolk, whilst at the same time the yolk content of the other cells decreases, so that at last, also with regard to the proteid yolk, a more uniform distribution throughout the embryo is achieved. Apparently, the proteins stored in insoluble form in the beta granules are mobilized in these late cleavage stages and are then able to pass through the cell walls. Normal distribution is, however, not achieved before the early trochophore stage.

Summarizing, it may be said that the cell walls form no absolute bar to a regrouping of the substances of the egg, so that, generally speaking, in eggs centrifuged earlier or later a more or less normal distribution of substances is achieved. Apparently, the factors responsible for the localization of the substances in normal eggs remain active in centrifuged eggs until a late stage, thus returning the substances displaced by centrifugation to their normal positions.

3. *Influence of calcium salts*

The first indications that the development of *Limnaea* is influenced by the concentration of $Ca^{\cdot\cdot}$-ions in the medium were obtained in the experiments of Klomp (Raven & Klomp, 1946). In distilled water as well as in solutions of urea and sucrose anomalous cleavage occurred, in which the cleavage furrows do indeed cut through the egg, but the blastomeres afterwards do not, or only incompletely, flatten themselves against each other; no cleavage cavity is formed. In later stages the cohesion of the blastomeres is totally lost, so that (e.g. in sucrose solutions) a group of twenty to thirty loose blastomeres is formed, sometimes still held together by the vitelline membrane which, already at the first cleavage, begins to detach itself from the surface of the egg in the region of the cleavage furrow. This latter fact pointed to a change in the properties of the vitelline membrane in a calcium-free medium as the cause of the anomalous cleavage. When a small quantity of calcium chloride was added to the solutions beforehand, cleavage was normal.

The experiments were continued by Hudig (1946). She observed that in distilled water the vitelline membrane surrounding the uncleaved egg wrinkles and is thrown into folds. Furthermore, the first polar body is now formed inside the membrane, like the second. At first cleavage the vitelline membrane is taken inwards with the cleavage furrow. When this furrow is complete the blastomeres begin to flatten a little against one another, but only for a short time; soon afterwards they round themselves off again. At this moment the membrane in the cleavage furrow loses contact with the egg surface and forms a kind of bridge between the blastomeres.

In distilled water to which egg capsule fluid has been added the vitelline membrane shows a normal appearance, the first polar body forms outside the membrane and cleavage is normal. To a lesser extent the same result could be obtained by adding crushed eggs to the distilled water: the vitelline membrane behaves less abnormally, a normal cleavage cavity is formed.

By the addition of small quantities of calcium chloride the wrinkling of the membrane is reduced; the first polar body tends to lie outside the membrane in higher concentrations of calcium chloride. Normal cleavage can be obtained in 0·005–0·04 % $CaCl_2$ solutions. A survey of all the experiments showed that no strict correspondence exists between the behaviour of the vitelline membrane and the mode of cleavage. Therefore, it may be concluded that $Ca^{\cdot\cdot}$-ions not only influence the properties of the vitelline membrane but also those of the egg cortex proper.

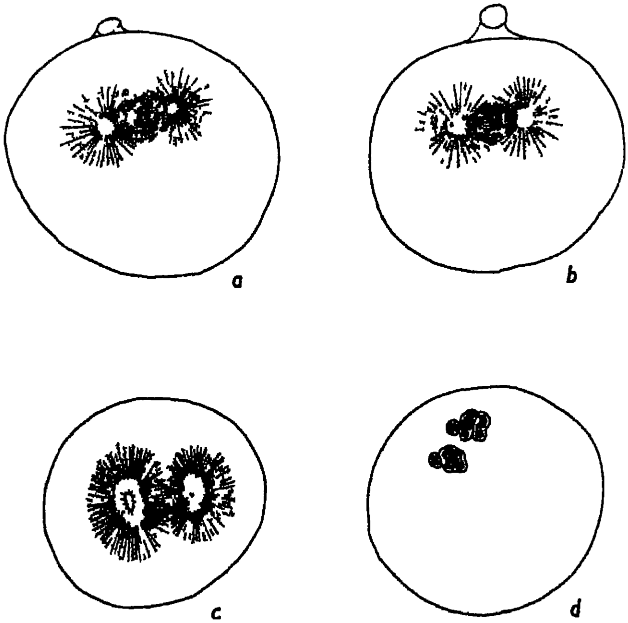

Fig. 9. Depolarization of second maturation spindle in eggs of *L. stagnalis* in 0·5 % $CaCl_2$. *a*, early anaphase; *b*, late anaphase; *c*, late anaphase, enlargement of asters; *d*, karyomere nucleus.

Finally, Mighorst studied the influence of higher concentrations of calcium chloride (Raven & Mighorst, 1946). The effects of the treatment of the eggs with concentrated solutions of calcium chloride are, in part, of a rather general nature. The eggs shrink by loss of water; in the higher concentrations this shrinkage may be followed by cytolysis. The development comes to a standstill; the stronger the solution employed, the earlier development stops. All these effects may be attributed to the hypertonicity of the medium.

As long as the solutions permit development, cleavage takes a normal course. When the eggs are subjected for a short time, immediately after oviposition, to still higher concentrations of calcium chloride, they may afterwards, upon return to distilled water, cleave normally. Evidently, they accumulate $Ca^{\cdot\cdot}$-ions from the surrounding solution, forming a reserve which suffices to warrant normal cleavage.

In a solution of 0·5 % $CaCl_2$, in some egg masses development is blocked during maturation. The first polar body is formed normally, but the second maturation spindle loses its contact with the animal pole, sinks into the interior of the egg and assumes a position at right angles to the egg axis (Fig. 9). The nuclear division may take place and leads to the formation of a group of karyomeres; it is not followed by a cell division. This aberration of development belongs to a class of phenomena known as 'depolarization'; the displacement of the maturation spindle expresses the fact that the forces working in the direction of the polar axis have been diminished in strength. In this connexion it is interesting that the ascent of the sperm aster and sperm nucleus to the animal pole, which is accomplished in normal eggs during and in the first hour after the maturation divisions, does not occur in these depolarized eggs. Hence it seems probable that the same factors which are, in normal eggs, responsible for the fixation of the maturation spindle at the animal pole attract the sperm aster and sperm nucleus to this pole too. Whether the accumulation of the animal pole plasm is due to the same attractive force could not yet be made out; further experiments are needed to answer this question (cf. below, p. 359).

4. *Influence of lithium salts*

The remarkable effects of a treatment with lithium chloride upon the eggs of *L. stagnalis* were discovered in 1942 (Raven, 1942). Egg masses, whole or divided into fragments, were placed in 0·01 and 0·001 % solutions (0·0023 and 0·00023 M, respectively) of lithium chloride in tap water during a period varying from half an hour to a few days; after this they were returned to tap water.

The results of this treatment may be of two kinds. In many experiments the embryos swell into large thin-walled vesicles, often pear shaped or dumb-bell shaped, filled with a transparent fluid. The wall consists partly of very flat transparent cells, almost free from yolk, partly of more or less irregular cells filled with yellow yolk granules. These formations bear a close resemblance to 'exogastrulae', such as develop from sea-urchin eggs under the influence of lithium. As a matter of fact, the study of these vesicles in sections has shown that their wall consists partly of ectodermal, partly of endodermal cells. The latter may differentiate into large vesicular cells with huge albumen vacuoles, closely resembling the albumen cells of the gut of normal embryos; they exhibit a peculiar apocrine secretion, by which the apical part of the cells is extruded into the external medium. In the ectodermal part of the wall large ciliary cells may develop. In the interior of the exogastrulae, mesodermal cells may be present; often, they have differentiated into structures resembling the protonephridia of normal embryos. As the germ layers in the exogastrulae show very abnormal relations to one another, these observations point to a relatively independent differentiation of these larval cell types.

In other instances embryos develop which resemble normal ones in many respects, but which exhibit a series of characteristic deformities. In one and the same egg mass a gradual series of malformations generally occurs, varying from almost or quite normal young snails to highly anomalous forms. In the mildest cases the dorsal part of the head is somewhat too narrow, and the eyes are closer together than in the normal embryo. One of the eyes may have divided itself into two parts. In other cases one of the eyes and the adjoining tentacle have been reduced, or these organs are wholly absent on one side. An increase in the lithium effect gives rise to embryos in which the eyes meet dorsally on the head or become wholly united, so that cyclopean embryos develop with only one eye placed medianly on the head. If the head region becomes still further reduced the eyes disappear altogether; the head is only a little lump over the foot. This, too, can be almost wholly absent. In all these cases the head region alone is involved in the reduction; the foot and the intestinal sac with mantle and shell may exhibit minor anomalies, but have fairly normal proportions. Finally, forms may arise with more prominent deviations, in which the various parts of the body are hardly recognizable.

The study of forty-two cyclopean, synophthalmic and anophthalmic embryos revealed that the nervous system is intact, as a rule, but the cerebral ganglia of both sides have often fused into a single unpaired mass which surrounds the cerebral commissure. The lateral tentacles have, in most cases, fused into a single median tentacle field. The head vesicle and velum and the anterior part of the apical plate have not been differentiated in a large percentage of cases; in all of these embryos, moreover, the differentiation of the posterior part of the apical plate has been suppressed (Raven, 1947). In normal embryos, this part of the apical plate consists of four big flat ciliary cells. According to cell lineage studies in *Planorbis* (Holmes, 1900) and *Physa* (Wierzejski, 1905), these are the cells $1a'''$–$1d'''$, i.e. the apical cells, surrounding the animal pole of the egg.

The fact that the head is formed from the cells lying in the neighbourhood of the animal pole, and that the suppression of the differentiation of the cells nearest this pole is the most constant deviation of development in these embryos, is not without significance. The characteristic action of lithium on *Limnaea* is, apparently, founded on a selective influence on material lying at the animal pole, or on processes which concentrate this material there. In echinoderms, as well as in amphibians, the action of lithium has been explained from the influence exercised on a gradient-field situated in the egg cortex (Dalcq, 1941). It is conceivable that a similar cortical gradient-field also plays a part in pulmonates.

Physiologically, the action of lithium is characterized by a high degree of inconstancy. The effect of the treatment depends on the concentration of lithium chloride and the duration of its action, but evidently individual differences in susceptibility between different egg masses play a part. Moreover, the stage of development in which the eggs are subjected to treatment is of paramount importance (Raven, Kloek, Kuiper & De Jong, 1947). There is a distinct maximum of sensibility for the production of exogastrulae shortly before and during the second cleavage. With regard to the production of head malformations, a first period of sensibility exists immediately after laying; it is followed by a period of minimum sensibility; then, the sensibility rises slowly to a maximum. In the period of generally reduced sensibility during the first three cleavages the eggs show an alternation of phases of increased and lessened sensibility corresponding to definite phases of cleavage. The second maximum of sensibility for the production of head malformations has been studied by Rijven (Raven & Rijven, 1948). Sensibility rises slowly and has a maximum 2–3 hr. after the eggs reach the 24-cell stage; then there is a sudden decrease, and 6 hr. later head malformations can no more be obtained by treatment with lithium chloride.

The action of lithium is not attended with loss of cell material; there can be no question of certain cells or groups of cells dying and being ejected. The fusion of the eyes into a single eye situated medianly or the absence of certain median parts of the head cannot, therefore, be accounted for by loss of cell material lying at the animal pole, but points to changes in the determination process. In the synophthalmic or cyclopic embryos the eyes are, apparently, formed from cell material that does not correspond to the material forming the eyes in normal embryos; a change has, therefore, occurred in the plan of organization of the head. It appears from the experiments that these anomalies of the head can still occur in eggs placed in lithium chloride not before the 24-cell or even the 32-cell stage; this proves that the determination of the organs of the embryo has not been unalterably fixed in these stages. This approaches these molluscs to the forms with 'regulative' development, and raises the suspicion that, generally speaking, the determination of development proceeds in both groups along the same lines.

With 0·001 and 0·01% LiCl solutions, no direct effects on the structure of the eggs can be observed. In order to determine these effects, A. P. de Groot (1948) has studied the influence of higher concentrations of lithium chloride on decapsulated eggs. With 4 and 2% solutions, the formation of the first polar body is suppressed. In 1·5 and 1% solutions, this is only the case when the treatment begins sufficiently early (about 30–35 min. before first polar body formation). In 0·6 and 0·5%, a first polar body is formed, but the second polar body is suppressed. In 0·4%, a second polar body may be formed, when the treatment begins less than 25 min. before first polar body formation. With lower concentrations, both polar bodies are formed; the percentage of eggs which undergo first cleavage increases and the retardation of cleavage diminishes with decreasing concentration. Below 0·3%, also second cleavages occur. Giant polar bodies are formed in a low percentage of cases in 0·4–0·2% solutions. In 0·6–0·2%, the eggs show a very intense amoeboid activity during and after the formation of the second polar body in the controls.

A comparison of these results with those obtained with solutions of calcium chloride and non-electrolytes shows that most of these phenomena may be accounted for by the osmotic pressure of the solutions. All lithium chloride solutions above 0·2% are hypertonic to the eggs. An excess pressure of about 10 atm. suffices to suppress the extrusion of the first polar body.

In 0·35–0·2% LiCl, the blastomeres formed at cleavage remain rounded and do not flatten themselves against each other; no cleavage cavity is formed. In 0·1 and 0·05 % flattening of the blastomeres occurs, and especially in the latter concentration quite normal 2-cell stages with cleavage cavity may be formed. This proves that the process of normal cleavage is not entirely dependent on the presence of Ca¨-ions, but that also in lithium chloride in optimal concentration normal cleavage is possible.

The cytological study of treated eggs gave the following results: in eggs treated with 1 % LiCl, the first maturation spindle sinks into the interior of the egg and degenerates; in its periphery, vacuoles appear in which small heavily stained rod-shaped bodies are situated. They show some resemblance to chromosomes; often they are present in pairs within a vacuole. In later stages, however, these bodies spread throughout the cytoplasm and their number and size increases considerably. Presumably, they are coagulation products of the cytoplasmic proteids, formed in consequence of the degeneration of the spindle. In the centre of the degenerating spindle, often a nucleus is formed by the egg chromosomes.

In 0·5 % LiCl, the first polar body is formed in a normal way; then, the maturation aster loses its contact with the animal pole and sinks into the interior; it degenerates, and again vacuoles with 'pseudocrystals' arise around this degenerating aster, whilst a nucleus-like structure may be formed in its centre. The subcortical plasm does not spread around the egg, but a gap remains at the animal pole. When the eggs are transferred to distilled water after some hours, abnormal spindle figures arise which are often multipolar.

In 0·4% LiCl, development may be deflected at various moments. The most interesting cases are those where, after normal formation of the first polar body, the egg chromosomes swell into karyomeres situated immediately beneath the animal pole in the remains of the vanishing maturation aster. No second maturation spindle is formed, but the sperm nucleus swells into a male pronucleus and rises to the animal pole which it may have reached already 35 min. after the formation of the first polar body i.e. 1–1½ hr. too early. Copulation of the pronuclei takes place, and cleavage spindles appear, which show, however, an abnormal orientation of the chromosomes. Evidently, in these eggs, after the extrusion of the first polar body, a condition is reached which in normal development does not occur before the end of the second maturation division. This condition leads automatically to the activation of the factors attracting the sperm nucleus to the animal pole. The premature formation of the male pronucleus may be considered as belonging to the class of events, called 'mise à l'unisson' by Brachet (1922).

In other eggs, treated with 0·4% LiCl, after the extrusion of the first polar body, a second maturation spindle is formed, but this sinks into the egg and places itself perpendicular to the egg axis. This is the phenomenon of 'depolarization' described by Raven & Mighorst (1946) in eggs treated with 0·5 % $CaCl_2$.

Depolarization phenomena may also occur at a later stage: both polar bodies are extruded, and karyomeres formed by the egg chromosomes, but these do not remain at the animal pole, but are shifted to a deeper position. In these cases, the migration of the sperm nucleus is delayed; evidently, a weakening of the directing factors of this migration has occurred.

Finally, in a further category of eggs treated with 0·4% LiCl, development proceeds normally till the copulation of the pronuclei at the animal pole, but then further development is inhibited.

In 0·2% LiCl the evolution of the nuclear and spindle apparatus proceeds normally. However, here a disturbance of cytoplasmic movements becomes very evident. The subcortical plasm shows a very abnormal distribution, great accumulations of this plasm occurring at various places. Furthermore, the formation of the animal pole plasm is altogether suppressed. The latter phenomenon occurs also at higher concentrations. In eggs treated with 1–0·2% LiCl, in no case has a distinct animal pole plasm been observed. On the other hand, the irregular distribution of the subcortical plasm occurs especially in 0·2% LiCl; in the experiments with higher concentrations only slight irregularities occur, most often in 0·4% LiCl.

As a 0·2% LiCl solution is nearly isotonic to the eggs, it is probable that this disturbance of cytoplasmic movements is a specific lithium effect. This conclusion is supported by the observation that in a 0·75% thiourea solution, which is likewise about isotonic to the eggs, a distinct formation of the animal pole plasm occurs (cf. below, p. 361).

Summarizing, it may be said that with higher concentrations of lithium choride, besides disturbances of the nuclear cycle which are probably due to the hypertonicity of the solutions, a specific disturbing influence on the distribution of the cytoplasmic substances has been observed. This points to a difference in the nature of the factors directing, on one hand, the evolution of the mitotic apparatus, on the other, the cytoplasmic movements. Further investigations will have to elucidate the true nature of both groups of factors.

5. *Influence of sodium sulphocyanate*

In sea-urchins and amphibians development is affected in a characteristic manner by sodium sulphocyanate. The effects of the action of this substance proved to be more or less opposite to that of lithium. A series of experiments has been carried out by Arendsen de Wolff-Exalto (1947) in order to find out whether this also applies to *Limnaea.*

Under the influence of 0·1% NaSCN the embryos showed an abnormal balloon-like swelling. This results from a disturbance in the water regulation. In extreme cases, the embryo becomes a large transparent balloon, which fills the egg capsule almost completely and enlarges it to two or three times its normal size. In most cases the organs are present in their normal forms and sizes, though often dislocated. No anomalies, pointing to a specific influence exercised on the process of determination, could be ascertained. The disturbance in the osmotic regulation comes into effect between the stages of trochophore and very late 'veliger' larva, and cannot be suspended any more later; thus the swelling has nothing to do with the formation of 'exogastrulae' in the lithium experiments.

In the embryos developing in a sodium or potassium sulphocyanate solution, at the 'veliger' stage a wine-red colour appears at special places. At first the eyes show themselves as red circles, two red bands appear underneath the head, and the mantle fold and foot acquire a red colour. In later stages the colour of the eyes gets darker, the tentacles, the oesophagus, and sometimes the nephridial canal, become pink; the stomach takes on an orange-red colour. The colour changes later into purple and dark brown, and at hatching it has almost disappeared. If the coloured embryos are taken out of the solution and further cultured in water the red colour disappears. So the substance causing the colour is presumably a product of metabolism, which cannot exist without a new supply of SCN′-ions. At first this colouring was supposed to be due to the formation of $Fe(SCN)_3$. However, no correspondence was found between the localization of non-masked iron and the appearance of the red colour. Therefore the chemical composition of this coloured substance remains in the dark.

6. *Influence of thiourea*

The influence of thiourea on the development of *Limnaea* has been studied by F. H. Sobels (unpublished). The eggs were treated either within the capsules or after decapsulation. Treatment of eggs within the capsules with a 1 % (=0·13 M) thiourea solution immediately after oviposition causes an inhibition of development. This is reversible when treatment has lasted 5 hr. or less, but at later stages development of the eggs is delayed as compared with the controls and mortality is high. When the treatment has lasted more than 5 hr., cleavage is very abnormal and development stops at an early stage.

The eggs of different egg masses show great differences in susceptibility, but as a rule all eggs of one batch are arrested in the same stage of development. Often they do not develop beyond the uncleaved stage after treatment with 1 or 2 % thiourea: the same can occur when decapsulated eggs are put in a 0·75 % solution. Mostly, however, the arrest of development occurs at the 4-cell or 8-cell stage. The 2-cell stage is found to be a critical phase: the eggs can still be arrested at the 4-cell stage by a treatment beginning during the first cleavage, and at the 8-cell stage by a treatment beginning at the 2-cell stage with a wide cleavage cavity; when the treatment is begun still later, development proceeds beyond these stages and mortality is rather low. At higher temperatures the susceptibility of the eggs seems to be greater. A study of the eggs arrested at the 4-cell or 8-cell stage shows that the nuclei are not always in the same phase. In some cases they have formed monasters with disorderly arranged chromosomes; in other batches development has stopped at the prophase stage or anaphase stage of division.

Eggs treated with thiourea show many abnormalities of cleavage. Even at the first cleavage the two blastomeres formed may be very unequal in size; at later phases 3-, 5- and 6-cell stages occur, the blastomeres show great divergences in size, and irregular groups of cells are formed having no resemblance to normal cleavage stages. In this abnormal cleavage the following points are of interest:

(1) The nuclei show various types of degeneration. In some cases cleavage

spindles with disorderly arranged chromosomes occur; sometimes the latter are widely scattered throughout the cytoplasm. Often pycnosis occurs, whilst monasters with irregular masses of chromosomes are also found.

(2) Cell division may be arrested. This can lead to uncleaved eggs with several groups of clotted chromosomes. At the second cleavage one of the cells may fail to divide, which leads to a 3-cell stage; at later cleavages the same phenomenon may occur in one or more of the blastomeres. As a rule these cells with arrested division contain two nuclei; apparently when a mitosis is not followed by cell division no further mitosis takes place.

(3) In many cases anachronisms of cleavage occur. At the second cleavage one of the blastomeres may divide much later than the other, so that a temporary 3-cell stage is intercalated between the 2-cell and 4-cell stages.

(4) Most interesting are deviations in the position of the cleavage spindles, which have often been observed in these eggs. In one batch, which had been decapsulated and put into 0·75 % thiourea immediately after oviposition, no cleavage occurred; the eggs were fixed after 8 hr., when the controls were in the 8-cell stage. The maturation divisions had taken place normally; two polar bodies were present in all the eggs. The animal pole plasm had been formed beneath the egg cortex of the animal side. The cleavage spindle had formed, but from this moment the development had taken an abnormal course. The asters of the cleavage spindle grew to enormous sizes; the spindle itself enlarged and elongated, so that in many eggs the asters were pressed against the egg cortex at opposite points of the surface. The chromosomes lost their regular arrangement and lay scattered over the whole spindle and outside it in the cytoplasm. Most interesting is, however, the fact that in many of these eggs the spindle rotated into the direction of the egg axis; one of the asters was situated at the animal pole, pushing aside the animal pole plasm, the other lay at or near the vegetative pole. Among seventeen eggs this occurred in four cases; in four other eggs the spindle had an oblique position intermediate between that described above and its normal position perpendicular to the egg axis.

Rotations of cleavage spindles may also occur at later stages. At the second cleavage one of the spindles may place itself perpendicularly to the plane of first cleavage, which leads to a T-shaped 4-cell stage. Sometimes both cleavage spindles take this position, which gives rise to a cleavage stage with 4 cells in a line. Most interesting, however, is the fact that in the embryos in which cleavage is delayed in one of the blastomeres of the 2-cell stage, the spindle of this cell rotates in such a way that it comes to lie parallel to the egg axis; in this way 4-cell stages are formed with a tetrahedric position of the cells.

These spindle rotations may be explained by a weakening through the action of thiourea of the factors governing the positions of the spindles in normal development. However, many of these cases demand another explanation. It must be remembered, that in the eggs described above, where the first cleavage spindle rotated into a vertical position, development had been delayed for a considerable time; in the controls the third cleavage had meanwhile taken place. It might be supposed that the conditions governing the succession of spindle positions in normal development change independently of other developmental processes, so that in these eggs which have missed out two cleavages the spindles are forced into a position corresponding to that of the third cleavage. This explanation, which can also be applied to those cases described above, where a delay of the second cleavage in one of the blastomeres causes the spindle of this cell to rotate into the position of a third cleavage spindle, agrees with similar phenomena observed by Hörstadius (1928) in sea-urchins. New experiments are planned to test this hypothesis.

V. DISCUSSION

The foregoing pages have been confined to a description of the developmental phenomena in *Limnaea*. We shall now discuss the results obtained in relation to the causation of development in other molluscs, and in other animals with spiral cleavage (Spiralia), that is annelids, nemertines and polyclads.

According to the classical view the eggs of the Spiralia are so-called 'mosaic eggs', which means that they show a precocious determination of the cells. Already at early cleavage stages, or even in the uncleaved state, the fate of the parts would be definitely and irrevocably determined. This view has arisen particularly from results of experiments on the development of isolated blastomeres, as made by Wilson (1903), Zeleny (1904) and Yatsu (1910) in nemertines, by Wilson (1904*a*) and, more recently, by Hatt (1932) and Costello (1945) in annelids, and by Crampton (1896), Wilson (1904*b*) and Conklin (1912) in molluscs. These experiments have shown that, in general, an isolated cell differentiates as it would have done in normal development, and that the removal of particular cells leads to the formation of defective larvae, lacking the parts which would have been formed by these cells in normal development. They gave rise to the idea that the cytoplasm of these eggs is a mosaic of different substances, the 'organ-forming substances'; during cleavage, which proceeds in these forms in a strictly regular, determinate way, these substances would be distributed passively among the blastomeres, which would exhibit, therefore, from the beginning, differences in their material composition and developmental potentialities. Experiments on the development of fragments of uncleaved eggs, such as those of Wilson (1903), Zeleny (1904) and Yatsu (1910) with *Cerebratulus*, and of Wilson (1904*b*) with *Dentalium* seemed to prove that these 'germinal localizations' are already present in the uncleaved egg.

A difficulty arose when it became necessary to reconcile this view with the results of centrifuge experiments, which showed that in many cases, where the structure of the egg had been entirely disturbed by centrifuging, nevertheless normal development took place. Such was, for instance, the case in *Cumingia* (Morgan, 1910), *Physa* and *Limnaea* (Conklin, 1910; Clement, 1938), and in *Crepidula* (Conklin, 1916, 1917). The fact that the displacement of the visible inclusions of the egg by centrifugal force did not interfere with normal development could only be explained by the assumption that these inclusions play no part in the determination of the cells;

in other words, the 'organ-forming substances' would not be displaced during centrifuging.

In many eggs of Spiralia particular cytoplasmic differentiations have been found at the polar regions of the egg: the polar plasms. An animal pole plasm has been found, for example, in *Nereis*, *Sternaspis*, *Arenicola*, *Podarke*, *Physa*, *Limnaea*, *Planorbis*, *Limax*, *Crepidula*, *Cumingia*, *Fulgur* and many Hirudinea and Oligochaeta. Vegetative pole plasms have been described in Oligochaeta and Hirudinea, and further in *Dentalium*, *Cumingia* and the annelid *Chaetopterus*. In many cases this plasm protrudes rhythmically during the first cleavage divisions, forming a so-called antipolar lobe; this occurs, for example, in *Chaetopterus*, *Sabellaria*, *Dentalium* and *Ilyanassa*.

Wilson (1904*b*) studied the development of eggs of *Dentalium* in which the antipolar lobe had been removed at the first or second cleavage. After removal of the first antipolar lobe the eggs developed into defective trochophore larvae, in which the post-trochal region of the body, the mesodermal bands and the apical tuft were missing. Removal of the lobe at the second cleavage yields larvae without the post-trochal region and mesodermal bands, but possessing an apical tuft. From these experiments it can be concluded that the vegetative polar plasm in these eggs plays an important part in development and contains the factors for the determination of particular structures of the embryo. Similar results have been obtained by Hatt (1932) in the polychaete *Sabellaria*.

Likewise, the experiments of Penners (1925) prove that in the oligochaete *Tubifex* the formation of the germinal bands, which produce the embryo, is dependent on the presence of the polar plasms, which fuse with each other in the cell *D*.

These results have led to the view that the polar plasms in the eggs of the Spiralia in general are to be considered as 'organ-forming substances', which by their distribution among the cells during cleavage determine the fate of the cells.

However, in recent years some observations have been made which raise some doubts on the exactness of the classical view. In the experiments of Hörstadius (1937) on the development of isolated cells and cell groups of the nemertine *Cerebratulus*, the latter usually develop according to their prospective fate (normogenesis), but in some instances a paragenetic development takes place, showing that the determination of the cells is not entirely stable and that they possess potentialities which do not usually become manifest.

Penners (1936, 1938), in later experiments, observed that the later development of *Tubifex* is not so strictly determinate as appeared from his earlier work. Indeed, the mesodermal parts of the body seem to have an important influence on the development of the ectodermal and endodermal organs.

Peltrera (1940), experimenting with the eggs of the mollusc *Aplysia*, found that in many instances isolated blastomeres, blastomere groups or egg fragments could develop into small but complete larvae; he concluded that the eggs of *Aplysia* resemble in many respects eggs of the 'regulation type' of development.

Our own experiments on *Limnaea* again deal a serious blow to the idea of a very precocious determination in the eggs of Spiralia. It has been shown that the pattern of determination of the head in *Limnaea* can be altered entirely by the influence of lithium as late as the 24-cell or even 32-cell stage. In the embryos of the cyclopean series, resulting from this treatment, the cells surrounding the animal pole, which form the posterior part of the apical plate in normal development, differentiate in quite another way and take part, for example, in the formation of the tentacle field or the cyclopean eye. This means that the pattern of determination of the head organs has not yet been irrevocably laid down even at this stage of cleavage.

It might be objected that our results are not quite comparable with those of previous experiments on blastomere isolation. In most cases the latter give information on the determination of larval organs alone, whereas our experiments concern the formation of adult structures. It might be supposed that for larval organs the classical view of a precocious determination is correct, whereas the determination of the adult organs might take place at a later stage. However, it has to be borne in mind that in the head of the *Limnaea* embryo a mosaic of adult and larval organs exists, and that in the origin of a cyclopean malformation both are concerned; to be sure, the central event of this process is the suppression of the larval 'apical plate' organ and its substitution by the adult organs 'tentacle field' and 'eye'.

Many attempts have been made to determine the chemical nature of the 'germinal localizations' by cytochemical methods. Ries (1937) studied the distribution of vitamin C, glutathione and of various cell enzymes in *Aplysia*, *Pleurobranchaea*, *Chaetopterus* and *Nereis*. In some cases characteristic localizations were observed, for example of vitamin C in *Aplysia*; in other eggs and with other substances, however, no local accumulations could be found. Gersch & Ries (1937) extended these results to other eggs and other enzymes; clear differences between different regions were found in various mosaic eggs, with great variations, however, according to the animal forms. Reverberi & Pitotti (1940) studied the distribution of oxidases and peroxidases; in *Nereis* a distinct localization can be observed, the benzidine peroxidase passing especially to the first somatoblast, whereas the indophenol oxidase gets into the second somatoblast. In the polychaete *Hydroides*, however, no definite localization of the two enzymes can be observed at early stages. Lehmann (1941) found the pole plasms of *Tubifex* to give an elective indophenoloxidase reaction.

Peltrera (1940), in his above-mentioned experiments on *Aplysia*, observed that an aberrant distribution of the egg substances among the blastomeres did not lead to the production of localized defects in the larvae, but rather to discordances in the relative sizes of organs. The egg substances do not represent definite 'organ-forming substances', but the development of the parts of the embryo is governed by 'cytochemical equilibria' in which various substances play a part.

Our observations on *Limnaea* agree with this point of view. The various plasms of the egg (vegetative and animal pole plasm, inner plasm, ecto- and endoplasm) do not exhibit absolute differences in their chemical composition, so far as could be made out with our present cytochemical methods. They show, however, slight differences in the relative abundance of certain substances: fat, mitochondrial lipoids, ribonucleic acid, sulphydril compounds. It seems probable, therefore, that the 'germinal localizations' are founded on relative rather than absolute differences between the territories of the egg.

A new light has also been thrown on the results of centrifuge experiments. With the eggs of *Nereis*, *Chaetopterus* and *Aplysia* (Raven, 1938), I observed that in many cases the substances displaced by centrifugal force do not remain in their new positions, but are redistributed throughout the eggs and assume a normal position with respect to the egg axis in a relatively short time. In those cases, however, where the distribution of the egg substances among the cleavage cells remained abnormal, a defective development ensued. Therefore, I expressed some doubts on the correctness of the conclusions drawn from previous centrifuge experiments.

The rapid redistribution of the displaced substances after centrifuging in *Aplysia* has also been found by Ries (1938) and Peltrera (1940), and our experiments showed the same thing in *Limnaea*. When the eggs are centrifuged soon after being laid, their structure often becomes nearly normal again before cleavage begins. When they are centrifuged at a later time, the redistribution of the substances is not entirely inhibited by the formation of the cell walls, but can go on even after that. Therefore, centrifuge experiments give no information as to the importance of these substances in the determination of cells. The proteid yolk of the egg alone is a rather inert substance; its unequal distribution among the cleavage cells does not interfere, however, with normal development. So, with respect to this substance, our results corroborate the conclusions drawn from earlier centrifuge experiments.

These observations directed attention to the factors responsible for the redistribution of substances after centrifuging. It may be presumed that the same factors govern the localization of the egg substances during normal development.

Spek (1930, 1934) studied the composition of *Nereis* and *Chaetopterus* eggs by means of vital stains having the properties of pH indicators. Beginning some time after maturation or first cleavage, the animal half of the eggs stained with a tint corresponding to an alkaline reaction of the cytoplasm, whereas the vegetative half took on a colour as if its reaction were acid. According to Spek the cytoplasm of the egg is at first a mixture of positive and negative colloids; in the fertilized egg the particles of opposite charge would be separated and accumulate at each pole of the egg. This would be due to differences in electric charge of the egg surface in consequence of local differences in permeability to the ions of the external medium; in this way an electric field would arise, in which the colloid particles would be shifted to opposite poles; hence, according to Spek, it is a 'kataphoresis in the living cell'.

Ries & Gersch (1936) observed the same phenomenon in *Aplysia* eggs. A 'bipolar differentiation' cannot only be demonstrated by the use of pH indicators, but also with rH indicators. However, they express some doubts as to the interpretation of the observed phenomena; according to them the latter would not be due to a separation of colloid particles, but rather to an accumulation of the proteid yolk in the vegetative half of the egg. The 'bipolar differentiation' is not specific for 'mosaic eggs'; it occurs also in eggs of the regulation type, whereas, on the contrary, several 'mosaic' eggs do not show the phenomenon (Gersch & Ries 1937; Gersch 1939).

By combined centrifugation and vital staining experiments I was able to show that the interpretation of Ries & Gersch is correct (Raven, 1938). The proteid yolk stains with an acid colour and by its shifting to the vegetative pole causes the 'bipolar differentiation' of the eggs of *Nereis*, *Chaetopterus* and *Aplysia*.

The cytoplasmic movements studied in the above-mentioned cases show, clearly, a close relation to the polar axis of the egg. The same is true of the cytoplasmic displacements found in *Limnaea*. The accumulation of the vegetative pole plasm, its subcortical extension to the animal side, the formation of the animal pole plasm and the fusion of both plasm substances at the animal pole at the third cleavage, followed by its unequal distribution to the cells during subsequent cleavages, are all related to the direction of the polar axis. We may conclude, then, that the factors directing these cytoplasmic movements are also related to the poles.

It had already been observed in older centrifugation experiments that the maturation spindle displaced by centrifuging has a tendency to return to the original animal pole of the egg (Lillie, 1909; Morgan, 1910; Conklin, 1916, 1917). This speaks strongly in support of the hypothesis that the factors governing egg polarity reside in a relatively immovable part of the egg. Probably this is the outer layer of the cytoplasm, the egg cortex. Recent experiments have made it very likely that the directing factors of the cytoplasmic movements are also located in the cortex. Thus, Lehmann (1940) has shown that the accumulation of the pole plasms in *Tubifex* is due to attractive forces exerted by the cortex at the poles. The same thing can be deduced from the fact that the animal pole plasm of *Limnaea* accumulates in centrifuged eggs beneath the cortex at the original animal pole, irrespective of the direction of centrifuging.

The experiments on the influence of lithium in *Limnaea* yield further information on this system of cortical factors. They have shown that in *Limnaea* there exists a polar gradient field (Raven, 1943), having a maximum at the animal pole. Lithium has a depressing action on the higher region of this field. Furthermore, our recent experiments seem to show that the factors constituting this polar gradient field are of a double nature. Those factors that govern the nuclear processes of maturation and fertilization are susceptible to hypertonicity, whereas the factors which direct the cytoplasmic movements are affected in a more or less specific way by lithium ions.

Summarizing, we may say that, contrary to the classical view of a rigid mosaic of pre-localized cytoplasmic substances in the Spiralia egg, the experiments of recent years have brought to light the importance of the directing factors of cytoplasmic movements, which bring about the localization of the egg substances during early development. The old static concept begins to give place to a more dynamic one which is in tune with our ideas on development in other groups of animals.

VI. SUMMARY

1. A method is described for obtaining egg masses of *Limnaea stagnalis* at the moment desired, and the nature of the egg-laying stimulus is analysed.

2. The oogenesis of *Limnaea* has been studied in detail, with special reference to the formation of various egg substances.

3. The insemination of the eggs and the formation of the egg mass are described.

4. The development of the fertilized egg from oviposition until the trochophore stage has been studied; special attention has been given to the cytochemical changes in the egg and embryo. A distinction is made between the 'chemo-differentiation', consisting of a redistribution of preformed substances during early development, and the 'chemical differentiation', accompanying the histological differentiation. The chemical differentiation leads in all three germ layers to a separation into two types of cells, which specialize in different directions and are destined to form larval and adult organs respectively.

5. The osmotic properties of the egg have been studied. The recently laid egg is, on an average, isotonic with a 0·093 M solution of non-electrolytes.

6. With the aid of centrifuge experiments, the swelling of protein granules, the viscosity and tension at the surface of the eggs, their changes during the uncleaved stage and the first cleavages, and the influence of lithium and calcium chloride solutions on these physical properties have been studied. Eggs centrifuged during the uncleaved stage develop into normal young snails in a large percentage of cases. The redistribution of the egg substances after centrifuging has been studied. It is concluded from the experiments that this redistribution is caused by factors residing in the relatively unmovable egg cortex. Apparently the proteid yolk does not play an important part in the determination of the cells.

7. The normal cleavage of the egg is dependent on the presence of $Ca^{\cdot\cdot}$-ions, but these may be replaced by $Li^{\cdot}$-ions in optimal concentration. The $Ca^{\cdot\cdot}$-ions influence the properties both of the vitelline membrane and of the egg cortex proper.

8. By treatment with lithium chloride solutions both exogastrulation and cyclopean malformations may be induced. For both types of malformation specific periods of susceptibility exist. In the embryos of the cyclopean series the differentiation of the most animal cells of the embryo is suppressed. It is concluded that lithium acts in *Limnaea* on a polar gradient field. Cyclopean malformations can still be induced at the 24-cell stage; evidently the pattern of determination has not yet been laid down irrevocably at this stage.

9. Recent experiments suggest that two groups of factors are involved in the conformation of the polar gradient field: (*a*) factors governing the evolution of the mitotic apparatus, which may be influenced by hypertonic solutions; (*b*) factors directing the cytoplasmic movements, which are susceptible to the influence of lithium.

10. Sodium sulphocyanate does not influence the pattern of determination in *Limnaea*. It causes a wine-red coloration, the nature of which is still obscure.

11. Treatment of the eggs with thiourea causes cleavage abnormalities. Cases of irregularities in cleavage, with deviations in the position of the cleavage spindles, suggest that the factors governing the succession of spindle positions in normal development change relatively independent of the other developmental processes.

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PRENATAL MORTALITY IN MAMMALS

By F. W. ROGERS BRAMBELL
Department of Zoology, University College of North Wales, Bangor

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I. INTRODUCTION

It is remarkable that so little is known about the amount and distribution of prenatal mortality in mammals. It is true that the problem is one of peculiar difficulty, yet comparatively little attention has been paid to it compared to that which has been devoted to the study of particular factors, genetical, physiological, or pathological, causing prenatal death. Even in man and the principal domestic animals, with which the bulk of the literature is concerned, information is almost confined to the mortality in late pregnancy, resulting in miscarriage or abortion, and at parturition, resulting in stillbirth. There is no satisfactory estimate of the total prenatal mortality from fertilization to birth for any single species of mammal, not excluding man. The purpose of this article is to examine critically the methods of estimating the amount and analysing the distribution of prenatal mortality in mammals in the light of the results of researches on wild rabbits in which I and my associates have been engaged for six years.* It is necessary also to examine thoroughly the nature and limitations of the data which can be obtained, from which all such estimates must be derived and on which their scope depends. It is not intended to attempt a systematic review of the literature but only to quote such data as are of comparative value in relation to the work on the rabbit.

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The work started as a general investigation of the reproduction of the wild rabbit (Brambell, 1944), but it was realized at an early stage that the prenatal mortality was unexpectedly heavy and that its distribution was especially interesting. It was apparent that, in particular, a large proportion of the litters was lost entirely on or about the 11th and 12th days of gestation (Brambell, 1942). It was felt that these initial results justified extension of the work with reference particularly to prenatal mortality. Consequently much more extensive data were collected and a more intensive investigation of the prenatal mortality was undertaken, the results of which have since been published (Allen, Brambell & Mills, 1947; Brambell & Mills, 1947 *a*, *b*, 1948).

The rabbit provides favourable material for work on prenatal mortality. First, the use of the wild animal enables us to examine the mortality in a population living under natural conditions and to obtain samples that are as near random as is possible, being biased only by the extent to which the method of obtaining them, by trapping, shooting or netting, is unconsciously selective. Secondly, they can be obtained in adequate quantities, since their destruction and disposal is organized, and they can be marketed after examination, thereby offsetting the costs of collection and reducing the expense to a minimum. Thirdly, they are large enough to enable all the information required to be obtained by macroscopic examination, thereby avoiding the labour of histological treatment and allowing much larger samples to be handled. Fourthly, they are polytocous, with large litters. This is important for two reasons. On the one hand, whereas the proportion of embryos lost and the proportion of litters suffering loss are identical in a monotocous species, they are not in polytocous animals and provide two measures of the prenatal mortality. These two measures are related, but the relationship varies according to the distribution of the mortality and hence provides a means of analysing that distribution. On the other hand, dead embryos are, as a rule, reabsorbed *in situ* in polytocous species, even if no other embryos in the litter survive, and abortion is relatively uncommon, whereas in monotocous species dead embryos are usually aborted. Reabsorption, being the slower process, results in the mortality being determinable for a much longer period thereafter than is the case with abortion, and it does not necessarily terminate pregnancy. Fifthly, the gestation period is short, thus ensuring that the critical early stages of pregnancy, which are comparatively uniform in duration in all mammals, will be found in a relatively large fraction of a random sample of pregnant uteri. Sixthly, the wild rabbit in this country has a prolonged and intensive breeding season throughout which material can be obtained. Finally, the tame races provide laboratory animals for experimental investigation of the problems raised.

II. OUTLINE OF THE OESTROUS CYCLE AND EMBRYOLOGY OF THE RABBIT

Understanding of the oestrous cycle and embryology is an indispensable preliminary to the study of prenatal mortality in any species of mammal, for the significance and limitations of the data cannot be assessed apart from this context, nor can an adequate plan for collection of the data be formed without it. A brief summary of the relevant facts concerning the rabbit will serve both to emphasize this point and to clarify much that follows. Fuller accounts of the oestrous cycle are available in the standard works (Marshall, 1922; Hammond & Marshall, 1925; Asdell, 1946).

Wild rabbits in Great Britain have a clearly defined and intensive breeding season extending from February to June inclusive, although sporadic breeding occurs throughout the year. Tame rabbits, kept under optimum conditions of temperature and nutrition, will breed continuously. Adult females come into oestrus at the beginning of the breeding season and, if they are unmated, oestrus persists indefinitely. A set of mature follicles is always present in the ovaries, ready to ovulate, throughout oestrus. These follicles, if they do not ovulate within 7–10 days, become atretic, often giving rise to blood follicles (Heape, 1905; Hammond & Marshall, 1925), but so long as oestrus persists they are replaced immediately by others. Thus successive sets of mature follicles maintain a continuous supply throughout prolonged oestrus (Hill & White, 1933; Smelser, Walton & Whetham, 1934).

Copulation or other strong sexual stimulation is necessary to cause ovulation, which occurs about 10 hr. thereafter (Walton & Hammond, 1928). The female will permit copulation at other times, although she is particularly receptive at oestrus. Wild rabbits copulate freely during November, December and January, when breeding is almost suspended, and also during pregnancy and pseudopregnancy (Brambell, 1944). As no vaginal plug is formed, the recent occurrence of copulation can be determined only from the presence of spermatozoa in the female reproductive tract. Ovulation terminates oestrus.

The corpora lutea are easily visible macroscopically throughout pregnancy. The newly ruptured follicles, which are about 1·0 mm. in diameter, are readily recognizable by the small pimples which the rupture points form on the surface of the ovary (Walton & Hammond, 1928). The corpora lutea develop gradually to a maximum of about 2·6 mm. in diameter at midpregnancy and do not begin to shrink until near full term, being about 2·0 mm. in diameter at the time of parturition. Thereafter they regress rapidly and disappear in a few days (Allen *et al.* 1947).

Gestation in tame rabbits lasts 30–32 days, being shorter in small than in large breeds, in wild rabbits 28–30 days (Southern, 1940). Pseudopregnancy, which lasts 16 days, follows ovulation if developing embryos do not implant, either through the ova being unfertilized or the embryos dying in the first 6 days. The corpora lutea persist throughout pseudopregnancy. At the end of pregnancy or pseudopregnancy the animal plucks fur and makes a nest, and the mammary glands contain colostrum.

A post-partum oestrous period immediately follows parturition or the end of pseudopregnancy. If the animal suckles and copulation does not occur oestrus subsides after a week and gives place to lactation anoestrus, which persists while active suckling continues. If fertilization results from the post-partum oestrus in tame rabbits which are suckling, the embryos die as a rule without becoming implanted. Occasionally, as for example when few young are suckled, pregnancy can proceed during lactation. In wild rabbits lactation does not interfere with gestation and, during the height of the breeding season, the majority of females are both pregnant and lactating simultaneously.

The ova, after their release from the follicles, pass into the funnel of the Fallopian tube, which embraces, but does not completely surround, the ovary. The majority enter the tube corresponding to the ovary from which they came, but some escape, and, migrating across the peritoneal cavity, may enter the tube on the other side. The ova are fertilized in the upper part of the Fallopian tube and then lose the cumulus of follicle cells with which, till then, they are surrounded. Cleavage occurs and an envelope of albumen is secreted around each ovum as it passes slowly down the tube. The ova reach the uteri as early blastocysts after 3 days and remain free in the uterine lumen for a further 3 days. During this time they become spaced out at approximately equal intervals in the uteri. Since the two uteri have separate cervical canals in the rabbit, there is no opportunity for the passage of embryos from one uterus to the other during this period. The blastocysts, at the beginning of the 7th day post-coitum, are spherical vesicles approximately 3·5 mm. in diameter, still surrounded by the stretched and attenuated zona pellucida, a hyaline non-cellular membrane, but occupying their definitive positions in the lumen with their embryonic shields orientated towards the mesometrium.

Implantation, which is of the central type, occurs early on the 7th day (Fig. 1 *a*). The zona pellucida is shed, exposing the trophoblast, which becomes attached to the uterine mucosa at a number of isolated points over the antimesometrial hemisphere. The blastocyst expands rapidly during the two succeeding days and attains a volume of 0·75 c.c. by the end of the 9th day, the increase of nearly seventy times in volume being due mainly to increase of the fluid content of the yolk-sac (Fig. 1 *b*). During this period the trophoblast of the bilaminar omphalopleur, over the antimesometrial hemisphere, has been invading and eroding the uterine mucosa. A new uterine epithelium is then regenerated beneath that invaded by the omphaloidean trophoblast and, by the 14th day, the old epithelium, together with the trophoblast and the entoderm of the bilaminar wall of the yolk-sac, have disintegrated (Fig. 1 *c*). Thus the cavity of the yolk-sac is opened up to the uterine lumen, exposing the entoderm of the splanchnic wall, which is thus in close proximity to, but not fused with, the regenerated uterine epithelium.

Meanwhile, the amniotic folds have been forming and closing over the embryo at the mesometrial pole. The chorionic trophoblast, covering the outer walls of these folds, becomes attached to, and invades, the mucosa of the placental ridges even before the closure of the amnion is completed on the 9th day, thus establishing the rudiment of the allanto-chorionic placenta. The allanto-chorionic placenta, which is of the haemo-endothelial type, is fully established by the 15th day (Mossman, 1926).

The age of embryos from the 8th to 20th days post-coitum can be determined by comparison with the normal table (Minot & Taylor, 1905). The embryonic heart has been observed to be beating at 9½ days. The embryos are orientated with their longitudinal axes at right angles to the axis of the uterus until about the 20th day, when they rotate through 90° (Reynolds, 1946). Prior to this rotation, the individual uterine swellings are subspherical and distinct, with the uterus constricted between each, but thereafter they merge, so that the uterus becomes cylindrical and equally distended throughout.

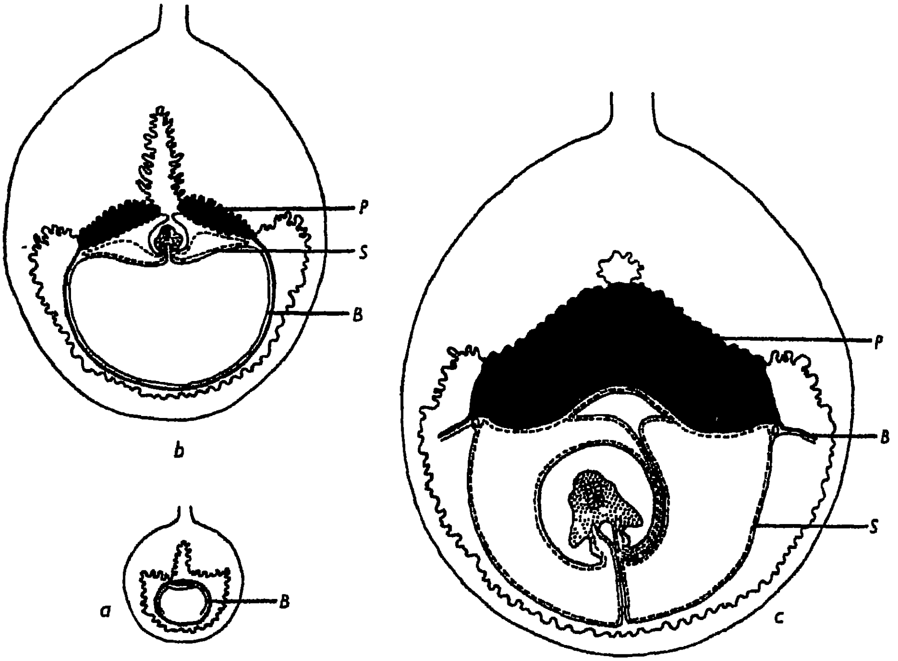

Fig. 1. Diagram of transverse sections of the uteri, embryos and membranes of rabbits at 7 days (*a*), 9 days (*b*), and 15 days (*c*) post-coitum (*c*. × 8). B, bilaminar omphalopleur; S, yolk-sac splanchnopleur; P, allanto-chorionic placenta. At 7 days the blastocyst is free in the uterine lumen and about to be become attached. At 9 days the size of the blastocyst has greatly increased owing mainly to the accession of yolk-sac fluid, the allanto-chorionic placenta is beginning to form and the amniotic folds are about to close. At 15 days the allanto-chorionic placenta is fully established and the bilaminar omphalopleur has disintegrated, opening up the yolk-sac cavity and exposing the yolk-sac splanchnopleur.

III. THE NUMBER OF OVA OVULATED AT OESTRUS

The size of litter at conception in a mammal is limited by the number of ova ovulated at oestrus. Some or all of these ova may be fertilized, but no more.

The average number of ova ovulated at each oestrus varies widely from species to species in mammals. Monotocous species produce as a rule only one ovum at a time. This is the case in man, in horses and cattle, in some of the bats, amongst the smallest of mammals, and in elephants and whales at the other end of the size scale. Polytocous species, on the other hand, normally produce a number of ova at a time. This is true of many rodents and insectivores amongst the smaller mammals as well as of pigs and other large species. Amongst the marsupials Hill (1910) records several instances in *Dasyurus* of 20–25 ova being ovulated at a time, two instances of 28, and one each of 30 and 35, and Hartman (1926) states that in *Didelphys* the average number is 22 and he records one instance of as many as 56. Amongst the placentals the insectivores display the greatest numbers. Bluntschli (1937, 1938) records up to 20 ova from each ovary in *Hemicentetes*, although the size of litter at birth does not exceed 10, and in Centetes he found 32 blastocysts on one occasion and 40 on another. Van der Horst & Gillman (1941) state that in Elephantulus about 60 ova are liberated from each ovary at a time, yet of these only one can develop in each uterine horn; the remainder are unfertilized or die at or before the 4-cell stage. The pig is the most prolific of the common domestic or laboratory animals, litters of 20 not being very infrequent, and Wentworth (1914) records an extreme case of 23 piglets in a litter. The Norway rat also is very fertile, for Perry (1945) records up to 18 corpora lutea, and the same number of implanted embryos, in wild individuals. Brambell & Davis (1941) record 19 corpora lutea in a pair of ovaries, and 17 implanted embryos in a pair of uteri of the multimammate mouse of Sierra Leone.

The number of ova ovulated can be estimated from the number of corpora lutea of the corresponding generation in the ovaries, because, normally, each corpus luteum represents a ruptured follicle from which one ovum was liberated. Errors in such estimates may arise from three causes; a single follicle liberating two or more ova, a follicle giving rise to a corpus luteum without liberating an ovum and from counting errors.

Polyovular follicles have been observed in many species (Hartman, 1926; Brambell, 1948) and are by no means uncommon in sections of mammalian ovaries. Obviously, such follicles might give rise to more than one ovum each, although, until recently, there was no direct evidence that they ever did so. Should they do so the number of ova ovulated would exceed the number of corpora lutea by the number of ova in excess of one each arising from such polyovular follicles. It has been shown that in the wild rabbit the numbers of embryos or implantation sites in the uteri may sometimes exceed the numbers of corpora lutea in the corresponding ovaries (Brambell, 1944). That this was due to polyovuly and not to a single ovum producing more than one embryo, by polyembryony, was shown by the absence of synchorionic twins. Identical twins in mammals, resulting from polyembryony, have a common chorion, presumably due to the persistence of the zona pellucida surrounding the ovum during cleavage preventing complete separation of the blastomeres. It was estimated, allowance being made for the expected proportion of animals in which polyovuly would be compensated by early prenatal mortality, that polyovuly occurred in 1·32% of litters of wild rabbits (Allen *et al.* 1947).

A follicle may give rise to a corpus luteum without liberating an ovum either through the ovum being retained within a ruptured follicle and so becoming enclosed in a developing corpus luteum or through an unruptured follicle transforming into a corpus luteum atreticum. The former will give rise to a corpus luteum of normal size with a rupture point, which can be distinguished only by microscopic identification in sections of the remains of the retained ovum. Unless a section of the corpus luteum which happens to pass through the retained ovum is carefully examined the fact that the ovum was not liberated would remain undetected. Although instances are recorded in the literature in the mouse, rabbit (Sobotta, 1896, 1897) and bat (O. van der Stricht, 1901), little information is available of the frequency of this phenomenon. Long & Evans (1922) were able to recover from the Fallopian tubes of 37 laboratory rats ova equivalent to 88·9 % of the corpora lutea in the ovaries and attribute the loss of 11·1 % mainly to retention in the follicles, but this opinion must be treated with caution in the absence of a count in sections of the proportion of corpora lutea containing retained ova. A corpus luteum atreticum, although also enclosing a retained ovum, is, as a rule, smaller in size than a normal corpus luteum and has no rupture point. These are formed sometimes at the same time as those resulting from an ovulation and, in some species, including the baboon (Zuckerman & Parkes, 1932), bank-vole (Brambell & Rowlands, 1936) and mare (Cole, Howell & Hart, 1931) they may arise during pregnancy. Histologically they may be difficult to distinguish from true corpora lutea. All the evidence suggests that in the wild rabbit they are formed only at the time of ovulation and from not more than 2 % of the mature follicles. It appears probable that large follicles, which regress before the time of ovulation, give rise to blood follicles and that only those which mature, but fail to ovulate, give rise to corpora lutea atretica in rabbits. A full discussion of the evidence and of the relevant literature has been provided elsewhere (Allen *et al.* 1947).

The error of counts of corpora lutea will depend on the peculiarities of the particular species involved, the method employed and the skill and experience of the observer. The amount which the corpora lutea normally project from the surface of the ovaries, their size and colour vary from species to species and affect the precision with which they can be counted. Counting is much more difficult in polyoestrous species with short dioestrous intervals, in which several successive sets of corpora lutea can be present simultaneously in the ovaries, as in the rat and the mouse, than it is in monoestrous species or those which only ovulate after copulation in which only one set of corpora lutea are present at a time, as in the rabbit. The reliability of counts made from the ovaries of pregnant animals will depend largely on whether the species is one in which the corpora lutea regress soon after mid-gestation, as in the human ovary, the horse, guinea-pig, cat, etc., or whether they persist until parturition before regressing, as in cows, sheep, goats, pigs, dogs, rats, mice and rabbits. Counts made from whole ovaries are less reliable, although much more rapid, than those made from slices or serial sections, since some corpora lutea may not be visible in surface view and others can be so closely apposed as to appear like one until sectioned. Finally, it requires much experience and practice to distinguish corpora lutea of different generations and corpora lutea atretica from true corpora lutea. The rabbit appears almost ideal material, as the corpora lutea project from the ovarian surface and are sufficiently large to be readily counted macroscopically, one set only is present at a time and during pregnancy the maximum size is maintained until full term, yet careful microscopic checking of macroscopic counts by experienced observers revealed a gross error of 6·4 % in the original counts, affecting 26·6 % of the pairs of ovaries. The major part of the error was due to the omission of 4·5 %

of the corpora lutea from the original counts. This error is substantial, and surprisingly large for favourable material. Counts of corpora lutea in successive 5-day age groups of pregnancy revealed that the mean number of corpora lutea in a set rises significantly at the beginning of pregnancy and declines significantly during the second half of pregnancy. This is difficult to account for other than as due to the omission from the counts of a larger proportion of small corpora lutea, both newly formed and regressing, than of those at the maximum size in mid-pregnancy, but the check counts on sectioned ovaries do not bear out such a distribution of the error. The fact that Corner (1923) observed a similar significant decline in the mean number of corpora lutea in pigs grouped according to the length of the embryos, which he was unable to explain satisfactorily, lends additional interest to the phenomenon. At least it is clear that the counting error must be estimated in all cases if reliance is to be placed on the results.

The distribution of the numbers of ova ovulated at oestrus, as shown by the numbers of corpora lutea of the same generation in the ovaries, has been investigated. The data for those species which produce large litters, such as the brown rat and the pig, do not deviate significantly from a normal distribution. The frequency distributions of other species, which produce smaller litters, are significantly positively skew, and the skewness tends to increase as the mean number of corpora lutea decreases. Moreover, the standard deviation tends to be a linear function of the mean, suggesting that the distributions form a family approximating to the normal as the mean increases. The investigation of these distributions would be of interest and might be of practical importance for the following reasons. Ovulation is an all-or-none response to a stimulus, presumably endocrine, and there is no apparent reason why the stimulus should not vary below the threshold necessary to evoke the response, especially in monotocous species in which the modal number of follicles ovulated at a time is one. Such anovular cycles do occur in some species at least, notably in man and the baboon, and may occur in others. It is possible that they may be found to account for an appreciable proportion of infertile matings in monotocous mammals. If the distribution of number of follicles ovulating at oestrus could be defined in terms of the mean then it should be possible to predict the proportion of anovular cycles in any species in which the mean could be estimated.

The tendency of a mammal to ovulate at oestrus a constant number of follicles, characteristic of the species, is known as the law of follicular constancy. It was demonstrated experimentally by the operative removal of one ovary, or of one ovary and part of the other. The remaining ovary or fragment of ovary in these circumstances undergoes compensating hypertrophy and then continues to produce approximately the same number of follicles at each oestrus as would have been expected from both ovaries, had they remained intact (Lipschütz, 1925; Lipschütz & Voss, 1925).

The follicles ovulated at oestrus are distributed at random between the two ovaries in polytocous mammals, and do not tend to be equally distributed between them. This is true of the mouse (Danforth & de Aberle, 1928), the bank vole

(Brambell & Rowlands, 1936) and the shrews (Brambell, 1935; Brambell & Hall, 1937) at least. Although it has frequently been claimed that in man and in other monotocous species the ovaries function alternately this is incorrect and the evidence that the distribution is other than random is unconvincing (see Brambell, 1948). The distribution of the embryos in the uteri need not correspond with that of the follicles from which the ova originated as migration of the ova or unimplanted blastocysts can occur by either of two routes. Newly liberated ova can migrate across the peritoneal cavity and pass down the Fallopian tube into the uterus on the other side, provided that the ovary is not completely enclosed in a sac from which the tube is the only way of escape. This external transmigration cannot occur in the mouse, which has closed ovarian capsules, but it can and does occur in rabbits. Out of 2179 litters of wild rabbits the number of implanted embryos on one side exceeded the number of corpora lutea in the corresponding ovary in 44 cases or 2·02% of the animals, involving a transmigration of 0·37% of the ova ovulated (Allen *et al.* 1947). Transmigration from left to right, or from right to left, appeared to be equally frequent. The only other possible explanation of the excess of embryos over corpora lutea on one side, with a corresponding deficiency on the other, would be the assumption of the production of more than one embryo from a single follicle on one side accompanied by a corresponding mortality on the other, and it has been calculated from the known incidence of polyovuly and prenatal mortality that this could not account for more than one-eighth of the observed transmigration. Internal migration of blastocysts directly from one uterus to the other can only occur in those species in which the uterine lumina of the two sides are continuous in the middle line and there is a common cervical canal. It was observed by Corner (1921) in the pig, and later confirmed experimentally by Warwick (1926). The latter eliminated the possibility of external transmigration by the excision of one ovary and the

Table 1. *Lateral distributions of corpora lutea and of implanted embryos of common shrews*

Difference of no. of corpora lutea or of embryos on the two sides	Corpora lutea			Implanted embryos		
	Expected $=m$	Observed	x^2/m	Expected $=m$	Observed	x^2/m
8+	0·4	0	1·35	0·1	0	8·10
7	0·9	1		0·3	0	
6	2·6	2		1·0	0	
5	5·7	3		1·9	0	
4	9·8	12	0·49	4·8	0	
3	16·6	19	0·35	6·2	0	6·20
2	20·6	18	0·32	11·5	8	1·07
1	27·3	28	0·02	10·6	19	6·66
0	13·1	14	0·06	7·6	17	11·63
Total	97·0	97	$\chi^2=2·59$ $n=5$ $P=0·8–0·7$	44·0	44	$\chi^2=33·66$ $n=4$ $P<0·01$

corresponding tube. The close approximation to perfectly symmetrical distribution of the embryos resulting from internal transmigration is shown clearly by the relative distributions of corpora lutea and of implanted embryos in common shrews (Brambell, 1935) summarized in Table 1. Whereas the distribution of corpora lutea does not differ significantly from a random distribution, that of the embryos does differ very significantly, as shown by the χ^2 test.

It is well known that the larger races of a given species tend to produce larger litters. Gregory (1932) showed that this is true for different varieties of rabbits, and that it is due to the number of ova ovulated being directly related to body weight. The relation appears from his figure to be linear. Using his values for the average body weights and average numbers of corpora lutea for each breed and weighting them by the numbers in each group, the regression of number of corpora lutea (Y) on live body weight in grams (x) is $Y = 1{\cdot}55 + 0{\cdot}00215x$.

The relation of the number of ova ovulated to the body weight has been found to hold also for populations of several species of wild mammals, including the bank vole (Brambell & Rowlands, 1936), the wild rabbit (Brambell, 1944) and the wild brown rat (Perry, 1945), and probably is of general application. Since size in these animals is closely related to age it is apparent that the number of ova ovulated must tend to increase with age also. It was found that in wild rabbits (Brambell, 1944) in Caernarvonshire the linear regressions of number of ova ovulated on cleaned body weight for the years 1941 and 1942 respectively differed significantly as regards the means but not as regards the slopes of the lines. Expressed otherwise, the mean number of ova ovulated at any given cleaned body weight was greater in 1942 than *in 1941, but the increase in the mean number of ova ovulated for a given increase in* body weight was the same in the two seasons.

IV. METHODS OF ESTIMATING THE TOTAL PRENATAL MORTALITY

The total prenatal mortality from fertilization to birth is very difficult to determine, *since it involves knowledge of both the numbers of ova fertilized and of the numbers* of young born alive subsequently. Clearly it is not practicable to obtain both these sets of data from the same animals, and hence recourse must be made to various indirect methods of estimating the one or the other. All these methods are open to objection and none is adequate in the sense of including the whole of the relevant information as to the loss both in litters that do, and in those that do not, survive to parturition.

The first, and perhaps the most valuable, method is to count the corpora lutea of pregnancy in the ovaries of animals that have recently given birth to litters of known size. This is an expensive method since in practice it involves sacrificing the mothers and the litters, and it is scarcely applicable to wild animals because mothers with new-born young are virtually unobtainable. It can provide no information regarding the mortality in litters in which none of the embryos survive to birth, nor does it enable loss of unfertilized ova to be distinguished from loss of fertilized ova. It does not distinguish the stages of development at which prenatal mortality occurs, although a careful examination of the placental sites in the uteri might provide this information, at least in some species. It can only be applied to those animals in which the corpora lutea persist throughout gestation and are countable in the post-partum ovaries. Nevertheless, the method is valuable because it does disclose the whole of the loss from ovulation to parturition in litters that survive, and it is to be regretted that it has been rarely employed. Crew (1925) records a small sample of 27 post-partum sows which had given birth to 213 young and contained 351 corpora lutea, showing a loss of 39·3% of ova ovulated in these animals.

Another method is based on statistical comparison of the mean numbers of corpora lutea in a set and of new-born young in a litter determined from different samples of the same population. This does not involve sacrificing mothers with new-born young, and it can be applied to species in which the corpora lutea do not persist throughout gestation, but otherwise it is subject to the same limitations as the first method in that it provides no information either as to the loss of entire litters before parturition or as to the stage at which loss occurs in the surviving litters. Moreover, since the number of follicles ovulated at oestrus varies with age, body weight, season and other environmental conditions, great caution is necessary to ensure that the samples are comparable on which the luteal and litter counts respectively are made; a precaution which has been overlooked in many instances in the literature. Some data for the laboratory rat are provided by Long & Evans (1922), who found a mean number of 10·8 corpora lutea in the ovaries and of 9·6 ova in the Fallopian tubes of 37 animals. They attribute this loss of 11·1% of ova mainly to retention in the ruptured follicles, but this opinion must be treated with caution. The mean number of young in a sample of 156 litters drawn from the same group of animals, was 6·4. There was thus a total estimated loss of 40·7% of ova, or of 33·3% if expressed as a percentage of tubal ova instead of corpora lutea, in litters in which at least some of the young survived to birth. Danforth & de Aberle (1928) found that the mean number of corpora lutea in 68 white mice was 8·4 and the mean number of newborn young in 500 litters 1–3 days old was 5·86, a loss of 30·24% of ova.

A third method is to determine the proportion of oestrous periods accompanied by copulation which result in live births. This method is not applicable to animals, such as the rabbit, which permit copulation at times other than oestrus. Like the two preceding methods this does not provide any information as to the stage at which loss occurs, or as to whether it is due to the ova being unfertilized or to subsequent prenatal mortality. Unlike the preceding methods, it does provide information regarding the proportion of litters which fail to reach full term, but it does not provide any information as to loss in litters that do survive. It should provide, therefore, a very valuable supplement to the other methods. It is not applicable to wild animals, since copulation cannot be observed, but it can be applied to the small murines in captivity, where the presence of a vaginal plug marks the occurrence of copulation as clearly as though it were seen. Parkes (1926*a*) provides data for 88 white mice with vaginal plugs of which 71 subsequently gave birth to litters, showing that 19·3 % of the litters did not reach term. It is applicable especially to farm animals, in which service is controlled and the lack of information regarding the loss in surviving litters is less important, since they are normally monotocous. Some examples of the numerous data of this kind in the literature for cattle and horses are summarized in Table 2. Data of fecundity in sheep, based on the proportion barren, are not comparable for two reasons. First, sheep ovulate more than one ovum as a rule, so that many may suffer some loss and yet may not be barren. Secondly, the ram is usually allowed to run with the flock and the services cannot all be observed. Animals which ovulate and copulate at oestrus and suffer early loss of the litter soon come into oestrus again, the mean length of cycle being only 16 days, and may then become pregnant. Animals which subsequently lamb and are therefore not included in the proportion barren may have lost one or more litters in this way. Hence the proportion of litters lost in sheep may exceed substantially the proportion barren. Heape (1899) provides data for 114,580 ewes, from 384 flocks, of which 4·5 % were barren. Nichols (1924) records 16,654 ewes belonging to several breeds, of which 3·4 % were barren, and of 606 cross-bred ewes of which 3·5 % were barren. Subsequently (1926) he records 3776 ewes, of which 4·2 % were barren. Combined data for five breeds gave 4·6 % barren, as compared to 8·3 % barren out of 253 yearlings of the same breeds.

Table 2. *Percentage of infertile services in cattle and horses*

Breed	Services	Percentage of services infertile	Authority
Dairy and beef cows	129	24·8	Gowan, 1918
Ayreshire cows	70	12·9	Corner, 1923
Clydesdale horses	28,241	47·5	Robinson, 1921
Thoroughbred horses	3,640	58·7	Robinson, 1921

The last method is based on comparison of the numbers of surviving embryos with the numbers of corpora lutea in animals at any stage of gestation after implantation. This method is the most widely applicable both to tame and to wild mammals, and by far the most valuable since it can yield information not only regarding both the loss of whole litters and the loss in surviving litters but also concerning the precise stage of development at which the loss occurs. Some estimates for various species, based on this method, have been extracted from the literature and summarized in Table 3. My data for wild rabbits are included for comparison. Henning's data for sheep are particularly interesting because he records the loss in animals with sets of 1, 2 and 3 corpora lutea separately, the values being 8·15 ± 1·28 for litters of one, 26·17 ± 2·55 for litters of two, and 42·86 ± 10·80 for litters of three. He states: 'Of necessity only those cases were studied in which there was persistent evidence of a pregnancy, that is, live or dead foetuses or remnants of foetal membranes. Consequently, cases of absence of fertilization or early embryonic death with complete resorption could not be included.' It follows that if all the embryos in a litter were lost early in development, which is quite probable, the author giving no indication of the relative frequency of 'dead' and missing embryos, then the animal, ceasing to be pregnant, would drop out of the sample. On a random basis the proportion of litters lost entirely would be inversely related to the number of corpora lutea. For example, if 40% of the embryos were lost and the loss distributed at random amongst the embryos irrespective of litter size and the chances were 1 in 10 of obtaining a dead embryo before it vanished then the apparent loss in surviving litters and dead litters that were recognized combined would be 37% in litters of three, 31% in litters of two and 6% in litters of one, figures which are not significantly different from those observed. Hence Henning's data can be interpreted as showing an overall loss of approximately 40% of the embryos, distributed at random irrespective of litter size. That this interpretation is not improbable will be apparent when the wild rabbit data have been analysed. It will be shown that gross estimates of the total mortality based on simple comparison of counts of surviving embryos and corpora lutea, such as are contained in Table 3, are worthless and even very misleading. Meanwhile, it should be noted that the estimate of 16·0% loss in wild rabbits derived in this way from the author's data falls well within the range of other estimates, although it is a false estimate which does not even approximate to the actual prenatal mortality.

Table 3. *Total loss both before and after implantation in samples of all post-implantation stages of pregnancy estimated from the numbers of corpora lutea and of healthy embryos*

Animal	Litters	Corpora lutea	Embryos	Loss (%)	Authority
Pig	22	396	267	32·6	Hammond, 1921
Pig	495*	4,480	3422	23·6	Corner, 1923
Pig	102	1,396	1028	26·4	Crew, 1925
Sheep	80	116	101	12·9	Hammond, 1921
Sheep	612	772	649	16·0	Henning, 1939
Ferret	89	919	652	29·1	Robinson, 1921
Stoat	12	135	103	23·7	Deanesly, 1935
Common shrew	49	353	320	9·3	Brambell, 1935
Wild brown rat	131	1,420	1135	20·1	Perry, 1945
White mouse		197	173	12·2	Parkes, 1923
Bank vole	70	321	286	10·9	Brambell & Rowlands, 1936
Multimammate mouse	15	207	185	10·6	Brambell & Davis, 1941
Grey squirrel	8	29	24	17·2	Deanesly & Parkes, 1933
Tame rabbit	56	577	391	32·2	Hammond, 1921
Wild rabbit	68	390	340	12·8	Hammond, 1921
Wild rabbit	1819	10,574	8877	16·0	Brambell

* Thirteen litters, including one with obvious uterine infection, in which all the implanted embryos were reabsorbing, are omitted as the author does not state the numbers of corpora lutea in these.

V. THE NATURE AND LIMITATIONS OF THE DATA

The problem of analysis of data of prenatal mortality was well stated by Robinson (1921) who wrote: 'Inquiry must be made as to the period at which prenatal death occurs, whether before or after fertilization, and if after fertilization whether before or after attachment of the zygote to the uterine mucosa.'

There is no easy method of distinguishing loss before, from loss after, fertilization. Some information can be obtained regarding the precise stages between ovulation and implantation at which loss occurs from degenerating ova or early embryos recovered by the perfusion of the Fallopian tubes or uteri. Unfortunately, degenerating ova or degenerating unimplanted blastocysts are recovered comparatively rarely, presumably because of their rapid elimination or disintegration.

Fortunately, the changes that take place in the uterine tissues at the sites where embryos have become implanted result in the formation of relatively persistent structures which remain recognizable even after the death of the embryos. These implantation sites therefore provide a convenient means of distinguishing between the loss occurring before and after implantation. Comparison of the numbers of corpora lutea with the numbers of implantation sites gives the loss before implantation. Comparison of the numbers of implantation sites with the numbers of surviving embryos gives the loss after implantation. Therefore the fractions of the mortality occurring in each of these two periods can be separated. Although Robinson (1921) clearly stated the need for inquiring into the period at which prenatal death occurs and whether 'before or after attachment of the zygote to the uterine mucosa' his own data do not permit of drawing the distinction, since he only gives the numbers of corpora lutea and of surviving embryos in litters of stated ages from ovulation to full term. Many authors make no attempt to distinguish; for example, Henning (1939), although providing counts of corpora lutea and embryos for 612 pregnant sheep, combines the loss before and after implantation under the provoking heading of 'Number of dead and living embryos'.

Although implantation sites are persistent structures, counts of them are liable to error as are those of corpora lutea. It was found that in wild rabbits (Allen *et al.* 1947) the mean number of implantation sites at successive stages of pregnancy from 11 days to full term rose significantly to begin with to a maximum of 5·419 ± 0·070 and then declined significantly to 4·923 ± 0·067 in the latest age group. The meaning of the initial rise is obscure, but it is tempting to attribute the subsequent decline to omissions of 9·2 % of the sites from the counts in the latest stages. It has been shown experimentally (Brambell, Henderson & Mills, 1948) that implantation sites in which the embryos died soon after implantation persist in a recognizable state until full term in litters in which some embryos survive. Nevertheless, such sites, though recognizable, are liable to be overlooked in uteri distended with embryos approaching full term.

The nature and limitations of the data for loss before, and of those for loss after, implantation are very different. Estimates of the loss before implantation, since they involve counts of implantation sites, are necessarily based exclusively upon pregnant animals in which implantation has occurred and in which, therefore, some at least of the embryos have survived to become implanted. Three limitations follow. First, litters in which no embryos survived long enough to become implanted are necessarily excluded from such data. They can provide no information regarding the incidence of the loss of whole litters before implantation. Secondly, since the loss that occurred before implantation is estimated from animals obtained after implantation has occurred, the data provide no information regarding the stage between ovulation and implantation at which the loss occurred. This is because degenerating ova or early embryos disappear rapidly, leaving no traces. Consequently the data do not permit of distinguishing between loss of ova through being unfertilized and loss of developing embryos. Thirdly, the data will include all the loss occurring before implantation in those litters in which some of the embryos survived to become implanted.

Fortunately, the number of litters lost entirely between the times of ovulation and implantation can be estimated in rabbits from the numbers of pseudopregnant animals obtained. The rabbit provides very favourable material for this purpose because ovulation in this species is not spontaneous and only occurs some 10 hr. after copulation. Hence all rabbits with recent corpora lutea in the ovaries must have copulated and must be either pregnant or pseudopregnant. Provided that all the pregnancies are identified as such, the remaining animals with recent corpora lutea in the ovaries must be pseudopregnant. Tubal ova and unimplanted uterine blastocysts can be recovered by perfusion in rabbits with a high degree of precision, thus allowing of the identification of early stages of pregnancy before implantation has occurred without the labour of serially sectioning the Fallopian tubes and uteri. This renders possible the routine identification of early pregnancies in large samples of this species, and is a very important, indeed an essential, technical consideration. Since the duration of pseudopregnancy in the rabbit is almost precisely half the duration of gestation, the proportion of litters lost entirely before implantation can be readily calculated from the proportion of pregnant to pseudopregnant animals obtained.

Estimates of the loss after implantation present entirely different problems. If estimates of the loss after implantation in litters that survive to birth could be based on counts of placental sites in the post-partum uteri and of newborn young in the nests, then the nature and limitations of the data would closely parallel those for loss before implantation, but this is impracticable. Instead it is necessary to derive them from counts of implantation sites and of surviving embryos in pregnant animals obtained at all stages of gestation from the time of implantation to parturition, that is from litters in which the loss is still in progress. Several limitations follow. First, such data cannot include the whole of the loss after implantation but only that part of it which has occurred before the death and autopsy of the mother; the amount of the total loss included will vary from animal to animal according to the stage of gestation at which it was killed. However, a fairly close estimate can be obtained from that fraction of the data which is derived from animals approaching full term. Secondly, the data cannot provide any information regarding the loss incurred at the time of parturition. Thirdly, the data will include litters in process of being lost entirely but in which at least one embryo still survived at the time of the death of the mother, and also litters with all the embryos surviving but which would have been lost entirely at later stages, had the mother survived. Fourthly, the data do provide information regarding the stage at which the loss occurs, both because the dead embryos are relatively persistent structures and are often sufficiently preserved to enable the age at which their death occurred to be determined, and because the data can be fractionated according to the age of the surviving embryos, and the loss estimated and compared in each of the successive age groups.

The estimation of the loss of whole litters after implantation in tame animals of which the history is known presents no difficulty, but it presents a very awkward problem with wild material of which the history is unknown. There are several possible methods, each of which involves some arbitrary assumption. One method is based on the relative frequency with which animals in early and in late stages of pregnancy are obtained. Thus if only 80 animals in late stages of pregnancy are obtained per day of gestation for every 100 per day of gestation in early stages, it follows that 20% of the litters have been lost between these two stages, provided that the sample is unbiased. Unfortunately, it is possible, indeed probable, that in the case of wild animals, trapping or any other available method of collection is liable to produce weighted samples. Another method is based on the relative frequency of litters in which all the embryos are dead and reabsorbing to those in which some or all the embryos are surviving at the time of the death and examination of the mother. The proportion of litters lost which the dead litters represent can then be calculated, provided that the duration of the process of reabsorption is known. We have tried to determine (Brambell, Henderson & Mills 1948) the duration of reabsorption in tame rabbits by experimentally killing all the embryos *in utero* at selected stages of development, either by injection of massive doses of stilboestrol or by direct surgical interference. It was found that the duration of reabsorption varies both with the age of the embryos at death and with the method of killing employed, but approximate figures were obtained. These could be used for the purpose of calculating the proportion of litters lost in the wild animals which the dead litters represented. Since abortion is a rapid process compared to reabsorption, and may even occur before the death of all the embryos in a litter, loss by abortion will not be included in estimates obtained by this method. There are some grounds for believing that abortion, as compared to reabsorption, may occur relatively frequently in rabbits after the 20th day of gestation. A third method is to determine from the distribution of the loss of embryos in surviving litters the proportion of these litters which are in process of being lost entirely, but this involves certain mathematical assumptions concerning the nature of the distribution.

It is evident, in view of these considerations, that the data of loss before implantation are subject to entirely different limitations to the data of loss after implantation, and that it is essential to separate these two sets of data before critical analysis can be attempted. That this treatment is justified is shown by the fact that the prenatal mortality suffered after implantation is distributed independently of that occurring before implantation (Allen *et al.* 1947). We have shown, to put it another way, that a rabbit which has lost some of the ova before implantation is no more or less likely to suffer further loss after implantation, than is a rabbit in which all the ova have survived implantation.

VI. THE LOSS OF OVA BETWEEN OVULATION AND IMPLANTATION

The loss of ova before implantation may be due either to the ova being unfertilized and consequently failing to develop, or to the death of developing ova, and it may result either in the loss of whole litters, if all the ova are affected, or to the loss of some only of the ova in litters in which other embryos survive to become implanted. It has been indicated already that litters lost *in toto* are automatically excluded from data derived from counts of corpora lutea and of implantation sites, since in them no embryos survive long enough to form implantation sites. Therefore the proportion of whole litters must be estimated from the proportion of animals that copulated and ovulated which contain neither ova developing normally nor implantation sites. The whole of the loss of ova in those litters in which at least some embryos become implanted will be shown by the counts of corpora lutea and of implantation sites, since this loss is complete once implantation has occurred. These two fractions of the total loss before implantation, being mutually exclusive and estimated from different data, will be considered separately. Consideration will be given then to the much more difficult problem of assessing the relative importance of non-fertilization as compared to death of developing embryos as causes of the loss.

It should be recognized that the distinction which is drawn here for polytocous animals between the loss of whole litters and the loss of some ova in surviving litters does not apply to animals which only ovulate a single ovum at oestrus, since the loss of this ovum is equivalent to the loss of the litter. Hence the distinction does not apply to monotocous species, except for the minority of ovulations when two or more ova are liberated, to which it could be applied and might yield valuable information. It follows that in monotocous species, such as cattle, horses and man, all the individuals which have copulated at oestrus (or the corresponding stage of the menstrual cycle in man) and which do not appear to become pregnant must either have had (*a*) anovular cycles, or have lost the ova either through (*b*) non-fertilization, or through the death of the embryos (*c*) before, (*d*) during or (*e*) shortly after implantation. The difficulty is to determine the relative importance of the fractions comprising the categories *a* to *e*, since the total infertility resulting therefrom is known to be large (for example, see Table 2 for cattle and horses). Much is known about factors which undoubtedly contribute to this loss, such as infecundity of the male, occlusion of the female tract, anovular cycles in man and cystic follicles in cattle, the precise time of copulation relative to ovulation, the condition of the cervical mucous secretion, etc., which are treated admirably by Hammond (1941) and in a recent symposium edited by Engle (1946); hence they need not be considered here. The important point is that the relative significance of the various factors cannot be assessed without a knowledge of the total loss, to which they contribute fractions, and this knowledge is lacking for monotocous mammals. Yet this information is vital and must be obtained before we can hope to formulate, much less to solve, the major problems of sterility in these mammals.

Very little is known even for polytocous animals regarding the incidence of loss of whole litters before implantation. Some data are available for wild rabbits (Brambell & Mills, 1947*b*). Since ovulation is dependent on copulation in rabbits, all females with corpora lutea in the ovaries may be assumed to have copulated. Those females with recent corpora lutea in the ovaries which have not got implantation sites in the uteri therefore will have either newly ovulated unfertilized ova or developing embryos not yet implanted in the tract, or else they will be pseudopregnant. Living ova or developing unimplanted embryos can be recovered with considerable precision by perfusion of the tract. A total of 882 animals with corpora lutea, but no implantation sites, were obtained, from 820 of which living ova or embryos 0–7 days post-coitum were recovered. The remaining 62 will have included all the pseudopregnant animals and any which may have had living embryos that the transfusion technique failed to recover. Since pseudopregnancy lasts approximately 16 days, that is twice as long as the interval from ovulation to implantation during pregnancy, these pseudopregnant animals represent 3·64% of the animals ovulating. Degenerating ova were actually recovered in 17, or 1·00% of the animals ovulating. Thus the loss of whole litters before implantation in wild rabbits was not less than 1·00% or more than 3·64%. It is not possible to determine what part of this loss is due to non-fertilization and what part to subsequent death of the embryos, but occlusion of the tract was actually observed in 4 animals, and is thus an appreciable factor. It is reasonable to suppose that non-fertilization may be much less important in animals, such as the rabbit and the ferret, in which ovulation is dependent on copulation and follows at an appropriate interval thereafter, than in animals in which ovulation is spontaneous and independent of copulation. It is interesting to note that the proportion of pseudopregnancies appeared to be greater in animals which had ovulated very small or very large numbers of ova, than in those with the more frequent litter sizes and also that it was higher at the beginning and end of the breeding season than it was at the height of the season.

Turning now to the loss before implantation in litters that survive, Robinson (1921) clearly recognized the need of knowing the stage at which loss occurs—'whether before or after fertilization, and if after fertilization whether before or after attachment of the zygote to the uterine mucosa'—but he does not appear to have realized that loss before and after implantation could be distinguished in post-implantation stages by comparison of the numbers of implantation sites with the numbers of corpora lutea on the one hand and with the numbers of surviving embryos on the other hand. Unfortunately, his data on ferrets are presented in such a way that these two fractions cannot be distinguished. He does record 74 ferrets killed at various stages between ovulation and implantation, which occurs 10–12 days after ovulation, which contained 696 corpora lutea and 572 ova or zygotes, a loss of 17·8% of ova. Since these data are derived from animals killed before implantation the loss will be incomplete and, in fact, the 34 animals killed from 5 to 10 days after ovulation had 341 corpora lutea and 235 ova or zygotes, a loss of 31·1% of ova, although still incomplete, as compared to 40 animals killed 1–5 days after ovulation with 355 corpora lutea and 337 ova or zygotes, a loss of 5·1 % of ova. Since this material was quite fresh and killed for embryological purposes it is unlikely that many living ova were overlooked. Hammond (1921) distinguishes between missing ova and atrophic foetuses and records the numbers of each in his data for pigs, sheep, tame and wild rabbits. Corner (1923), following Hammond, makes a similar distinction. Their data, together with others extracted from the more recent literature, including those for the wild rabbit, are summarized in Table 4. It will be seen that the lowest percentage loss observed was in sheep, wild rabbits coming next, whereas the highest was in tame rabbits. Danforth & de Aberle (1928) provide data for white mice which show that the mean number of corpora lutea in 68 litters was 8·4 and the mean number of living and dead embryos in 500 litters, examined mainly on the 12–18 days of gestation, was 6·35, which suggests a loss of 24·4% of ova before implantation. Their data are not included in the table because the two counts were not made on identical samples.

Table 4. *Loss before implantation in samples of all post-implantation stages of pregnancy estimated from the numbers of corpora lutea and of implantation sites*

Animal	Litters	Corpora lutea	Implanted embryos	Loss (%)	Authority
Pig	22	396	316	20·2	Hammond, 1921
Pig	495	4,480	3,485	22·2	Corner, 1923
Pig	102	1,396	1,117	20·0	Crew, 1925
Sheep	80	116	109	6·0	Hammond, 1921
Stoat	12	135	108	20·0	Deanesly, 1935
Wild brown rat	131	1,420	1,238	12·8	Perry, 1945
Grey squirrel	8	29	26	10·3	Deanesly & Parkes, 1933
Tame rabbit	56	577	441	23·6	Hammond, 1921
Wild rabbit	68	390	350	10·3	Hammond, 1921
Wild rabbit	2179	12,551	11,363	9·5	Brambell & Mills, 1947*b*

The data for the wild rabbits (Brambell & Mills, 1947*b*) were sufficiently extensive and complete to permit of analysis. Biologically it is obvious that prenatal mortality may result from failure either of the maternal or of the foetal organisms. Prenatal mortality resulting from maternal failure will tend to fall on the litters as units and consequently the proportion of litters lost will coincide with the proportion of embryos lost, as in a monotocous species. This type of loss has been estimated already from the proportion of pseudopregnant animals obtained, and is *ipso facto* excluded from the data derived from counts of corpora lutea and of implantation sites now under consideration. Prenatal mortality resulting from embryonic failure will tend to fall on the embryos as units. If the total loss of embryos is due to a large number of separate factors, genetical or otherwise, each causing a small part of the total loss, then it will tend to be distributed at random amongst the embryos. Such random loss of embryos will be distributed amongst the litters of size x in accordance with the expansion of the binomial $(p+q)^x$ where p = the proportion of ova lost, q = the proportion of ova surviving and $p+q=1$. Hence the proportion of litters suffering no loss would tend to be q^x, and the proportion of litters suffering loss $1-q^x$.

A third and intermediate type of relationship will exist if the probability of loss in some litters is greater than in others, for example, if some mothers provide an unfavourable but possible environment in which only the stronger embryos can survive, or through the enhanced probability of sibs inheriting the same lethal genes.

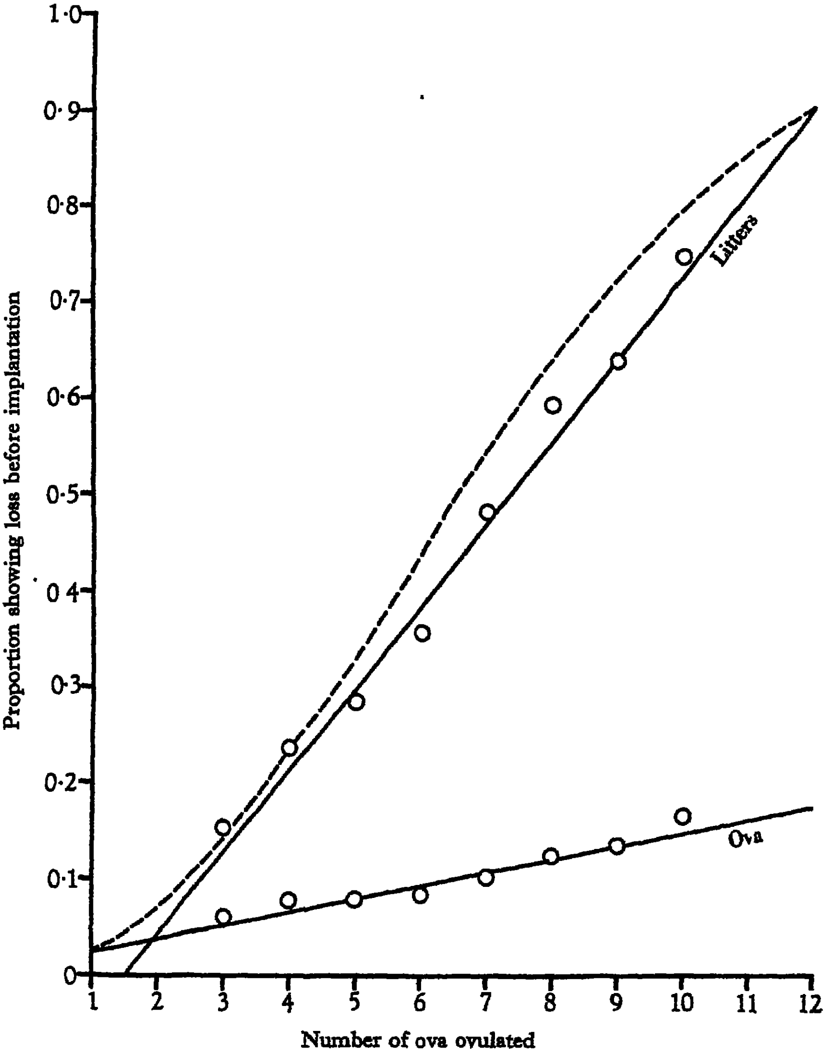

Fig. 2. Graphical representation of the proportions of ova lost and of the proportions of litters suffering loss before implantation in wild rabbits according to the number of ova ovulated. The circles represent the group means, the solid lines the empirical regressions and the broken line the expected proportion of litters suffering loss calculated from the proportion of ova lost on the assumption that the loss is distributed at random (from Brambell & Mills, 1947*b*).

Therefore it is profitable to examine both the relation of the proportion of litters suffering loss and the relation of the proportion of ova lost to the initial size of litter. In fact, both the proportion of litters showing loss and the proportion of ova lost vary with the initial size of litter, that is with the number of ova that were ovulated as shown by the number of corpora lutea. Linear regressions have been fitted to the data and are shown in the graph in Fig. 2, together with the group-means. It can be seen that the proportion of ova lost increases with the number of ova ovulated. It must be concluded, since the increase undoubtedly is significant, that the more fertile females, which ovulate the larger numbers of ova, provide a less favourable environment, either for fertilization of the ova or for their subsequent early development up to the time of implantation, than those which ovulate smaller numbers. The proportion of litters affected rises still more steeply with increasing size of litter and approaches the theoretical curve of distribution of mortality amongst the litters calculated from $1-q^x$, and shown as a dotted line in Fig. 2. Hence it is apparent that the greater part, but not the whole, of the mortality tends to be distributed at random amongst the litters and so must be due to factors which affect the embryos as independent units.

Although the observed proportion of litters suffering loss is only slightly less than the expectation for a random distribution, it is significantly less. This means that there is a tendency for the loss to be concentrated in some litters. Comparison of the observed and expected numbers of litters showing a loss of 0, 1, 2, 3 or more ova confirms this conclusion, for the observed number of litters showing a loss of three or more ova is significantly and substantially in excess of the expectation. The problem arises as to whether the loss, although tending to be concentrated in certain litters, is distributed at random amongst those litters that do suffer loss. It has been shown by further analysis that this is not so, but that on the contrary the sample of litters showing loss is not homogeneous, the probability of loss of ova being greater in some of them than in others.

The loss before implantation in litters that survive is less in animals above the average body weight than in those below it. It is less also in animals with milk in the mammary glands than in those without and which therefore were not lactating. No significant difference in the incidence of loss was detected either from place to place, or from year to year in the same place, or during successive months of the breeding season.

The two methods summarized above of estimating the loss before implantation are complementary and mutually exclusive, the one dealing only with the loss of whole litters and the other only with the loss of some ova in surviving litters. Both include the loss through non-fertilization as well as the loss through death of developing embryos, and neither provide a means of distinguishing between these two kinds of loss. The problem of estimating the loss through non-fertilization appears baffling at present, the only apparent solution being the almost prohibitively laborious one of detailed cytological determination of a sufficiently large sample of ova from freshly killed animals. Some light can be thrown by statistical means upon that part of the loss through non-fertilization which is due to occlusion of the Fallopian tubes or uteri. It has been shown above that an appreciable, though probably small, part of the loss of whole litters before implantation is due to this cause. Clearly occlusion of the tract could only operate on one side in the case of litters in which some embryos survive, since it would result in the loss of all the ova on that side. The problem is to determine statistically whether the loss of two or more ova is distributed at random between the two uteri of each pair or whether it tends to be concentrated on one side. It is necessary to know both the initial distribution of ova between the two sides, as shown by the number of corpora lutea in each ovary, and the numbers of litters showing a loss of 2, 3, 4, etc. ova to calculate the expected random distribution. Suppose that x is the initial size of litter, x' is the number of ova lost, x'' is the number of ova surviving, and a is the number of corpora lutea in the left ovary. Then the probability that n ova should be lost in the left uterus and hence $x'-n$ in the right uterus is $T'' \times T'/T$ where T is the $(a+1)$th term in the expansion of $(1+1)^x$, T' is the $(x'-n+1)$th term in the expansion of $(1+1)^{x'}$ and T'' is the $(a-n+1)$th term in the expansion of $(1+1)^{x''}$. The expectation of any given combination of x' and n can be obtained by multiplying the probability by the proportion of litters of initial size x which show a loss of x' ova, and by the number of these litters which are distributed with $(x-a)$ corpora lutea in the right ovary. A significant tendency to the concentration in one uterus of a pair of a loss of two or more ova was found in wild rabbits by this means (Brambell & Mills, 1947*b*). Out of a total of 2073 animals 92 had lost all the ova on one side but not on the other. Had the loss observed been distributed at random between the two sides then the expected number showing a loss of all the ova on one side would be 74. The observed excess of 18 is significant and represents 1·8 % of the animals. It can be attributed to factors which affected one tract only, of which occlusion is the most obvious. Occlusion of one side actually was observed when transfusing the tract in a few instances. Thus, although occlusion is the cause of an appreciable loss of ova on one or both sides in rabbits, it is obvious that it is a much less important cause of sterility in them than it is commonly supposed to be in man.

It has been shown above that in wild rabbits between 96·4 and 99·0% of litters survive until after implantation and that in these 9·5 % of the ova are lost. Hence the total loss of ova ovulated before implantation in litters that survive and those that do not is between 10·2 and 13·0%, a heavy rate of wastage for the first quarter of the gestation period alone. It is tempting to speculate in the light of the results as to the possible factors which may contribute to the loss. The increase in the proportion of ova lost in surviving litters with increasing litter size is surprising, since factors such as overcrowding of the embryos in the uteri or competition between the embryos for limited supplies of nutriment, which might be expected to become important at later stages, scarcely could be operative before implantation. Yet, somehow, the more fertile females, which produce the larger litters, provide a less favourable environment either for the fertilization or for the subsequent development up to the time of implantation of the individual ova. The relation of the loss to the body weight of the mother also indicates that the loss is affected by the maternal environment and is not due entirely to genetical or other factors intrinsic in the ova. Endocrine or nutritional influences suggest themselves. Yet the fact that the loss is less in animals with milk, which include all those suckling and which therefore are sustaining much greater nutritional demands, than in those which are not, is difficult to reconcile with nutritional deficiency. The idea of endocrine deficiency of the mother as a factor in prenatal mortality before implantation is more in keeping, since the animals without milk and which, therefore, could not have bred recently might be expected to include any with such a deficiency.

VII. THE LOSS OF EMBRYOS AFTER IMPLANTATION

The information available in the literature of loss after implantation is disappointingly small. Such data as are available are summarized in Table 5. Danforth & de Aberle (1928) found a mean number of 6·35 living and dead embryos in a sample of 500 white mice examined during the last third of gestation, and a mean number of 5·86 in 500 litters of newborn young 1–3 days old, indicating a total loss in surviving litters after implantation, including the loss at parturition, of 7·7%. These data are not included in the table since they are not comparable with the others. It will be observed that in all species except the sheep the loss appears to be less than before implantation. The author's data for wild rabbits provide an apparent mortality of 7·6%, which is almost midway between the extremes, but it will be shown that when these data are critically examined and analysed the real loss is very much heavier than appears from the crude percentage of dead embryos. Consequently, in the absence of such analysis, the other percentages contained in this table cannot be accepted as more than minimal and may be very much less than the true values. Corner (1923) alone, of the other authors quoted in the table, provides information as to the proportions of abnormal embryos at successive stages of pregnancy. His data are summarized in Table 6. The percentage of abnormal embryos is highest in the group in process of implantation and thereafter falls to a minimum in the 41–150 mm. group and rises again towards full term. Since prenatal mortality is

Table 5. *Loss after implantation in samples of all post-implantation stages of pregnancy estimated from the numbers of implantation sites and surviving embryos*

Animal	Litters	Implanted embryos	Healthy embryos	Mortality (%)	Authority
Pig	22	316	267	15·5	Hammond, 1921
Pig	495	3485	3422	1·8	Corner, 1923*
Pig	102	1117	1028	8·0	Crew, 1925
Sheep	80	109	101	7·3	Hammond, 1921
Stoat	12	108	103	4·6	Deanesly, 1935
Wild brown rat	131	1238	1135	8·3	Perry, 1945
Grey squirrel	8	26	24	7·7	Deanesly & Parkes, 1933
Tame rabbit	56	441	391	11·3	Hammond, 1921
Wild rabbit	68	350	340	2·9	Hammond, 1921
Wild rabbit	1834	9658	8927	7·6	Brambell & Mills, 1948,†

* Thirteen litters with all the implanted embryos reabsorbing are omitted.
† 164 litters with all the implanted embryos reabsorbing are omitted.

bound to be cumulative as pregnancy proceeds in any given population of pregnant animals, a decline in the apparent mortality must be due either to the disappearance of placental sites pertaining to embryos that have died in uteri in which other embryos survive, thus decreasing the apparent mortality in them, or to the death of all the embryos in some animals with litters which had suffered loss, and the consequent disappearance of these from the population of pregnant animals. The latter is the more probable explanation and, in the case of wild rabbits in which a similar phenomenon has been observed, is certainly the true one, as will be shown. It was, in fact, analysis of the mortality in successive age groups of litters, that directed attention first to the heavy loss of whole litters around the 12th day post-coitum (Brambell, 1942); a loss that was not apparent when all the data were combined (e.g. Table 3). This is an important principle and no estimate for loss after implantation based on counts of placental sites and embryos in surviving litters which fails to take it into account can be relied upon.

Table 6. *Loss after implantation in pigs*
(extracted from Corner, 1923)

Age group	Litters	Healthy embryos	Abnormal embryos	Implanted embryos abnormal (%)	Differences
151 mm. c/r to term	156	876	25	2·77 ± 0·55	2·19 ± 0·58
41–150 mm. c/r	212	1531	9	0·58 ± 0·19	2·20 ± 0·55
8–40 mm. c/r	127	1015	29	2·78 ± 0·51	3·60 ± 1·68
13–21 days (During implantation)	30	220	15	6·38 ± 1·59	

The loss after implantation in the whole sample of rabbits was 7·6% of the embryos, affecting 19·7% of the litters. Whereas the proportion of embryos lost did not vary significantly, the proportion of litters suffering loss varied directly with litter size. The fitted regression lines and group means are shown in Fig. 3. The expectation of litters suffering loss on the assumption that the loss of embryos is distributed at random amongst the litters is represented by the broken line. It can be seen at once that the observed proportion of litters suffering loss falls far short of this expectation, in marked contrast to the approximation of the observed loss before implantation to expectation, as is shown by comparison of Figs. 2 and 3. This indicates that a substantial part of the loss after implantation may be falling on the litters as units, whereas it has been shown already that litters lost as units are necessarily excluded from the data for loss before implantation. Let it be assumed that the loss after implantation is falling at random in part upon the litters as units and in part upon the embryos as units in the surviving litters, irrespective of litter size and, further, that the loss falling upon the litters as units results in the death of the embryos in each litter affected in sequence, not simultaneously. The proportion of dead embryos in those litters which are in process of being lost will tend to be

$l/2$, where l is the proportion of litters affected. It can be shown that the total mortality would then tend to be distributed as

$$E = l/2 + p(1-l), \tag{1}$$

and

$$L = lq^x - q^x + 1, \tag{2}$$

where E = the proportion of dead embryos, L = the proportion of litters showing loss, l = the proportion of litters in process of being lost as units, p = the proportion of embryos lost as units, $q = 1 - p$, and x = the size of litter at implantation. This theoretical distribution fits the data satisfactorily.

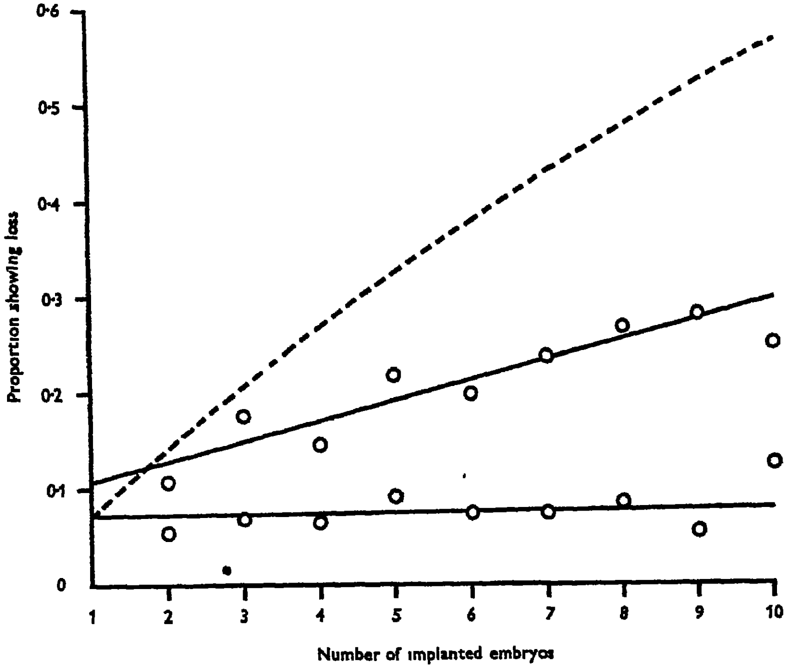

Fig. 3. Graphical representation of the proportion of embryos lost and of the proportion of litters suffering loss after implantation in wild rabbits according to the size of litter at implantation. The circles represent the group means. The solid lines are the empirical regressions. The broken line represents the expected proportion of litters suffering loss calculated from the proportion of embryos lost on the assumption that the loss is distributed at random (from Brambell & Mills, 1948).

The evidence derived from the surviving litters that some are in process of being lost entirely, is confirmed by the frequent occurrence of animals in which all the embryos were dead and autolysing and which, therefore, could not be classified as still pregnant. The embryonic remains were sufficient in 101 of the 164 such animals obtained, to enable the stage of development at which the last embryo had died to be determined and so to show that two-thirds of these litters were lost between the 11th and 15th days post-coitum inclusive. These data also enable it to be shown that the greater the number of embryos in the litter which become implanted, the less is the probability of the litter being lost.

Examination of the percentage of autolysing embryos and of the percentage of uteri containing them in the surviving litters for each day of gestation in Table 7 shows that both rise at first to a peak extending over the 12th–15th days inclusive, and then decline. It is obvious, for the reasons given above in relation to Corner's data, that the majority of the litters suffering loss before the 16th day must be lost entirely, so dropping out of the samples of later stages, and that these litters in process of being lost between the 12th and 15th days inclusive must represent earlier stages in the process of which the animals with all the embryos dead and autolysing are the end-point. The continued fall in the proportion of litters suffering loss from the 16th to 20th days inclusive is more difficult to explain because there are not enough animals with all the embryos autolysing at these stages to account for their loss by this means. Therefore, either some of the implantation sites in which the embryos had died must have been overlooked and omitted from the counts of later stages or some of these litters must have been aborted, not autolysed *in situ*. There is, in fact, some collateral evidence to support both these explanations and it is not possible at present to decide between them.

Table 7. *Loss after implantation day by day in wild rabbits*

Age (days)	Litters	Embryos	Litters suffering loss (%)	Embryos lost (%)
9	78	408	1·28	0·25
10	74	414	9·46	4·11
11	64	368	32·81	11·14
12	63	318	57·14	27·36
13	88	479	57·95	29·02
14	66	345	54·55	26·67
15	44	225	56·82	26·67
16	111	607	39·64	15·98
17	96	513	28·13	9·16
18	80	446	22·50	4·71
19	41	225	29·27	7·56
20	30	165	26·67	7·88
21–25	303	1598	8·91	2·25
26–32	450	2235	5·11	1·16

The difficulty of estimating the actual proportion of litters lost as units after implantation has been mentioned. Using the methods outlined on p. 385, an estimate of 35·5% of litters lost after implantation is derived from the relative frequency of animals obtained in early and late stages of pregnancy. An estimate of 35% is derived from the relative proportion of living to dead litters obtained, allowing for the probable rate of disappearance by autolysis of dead litters, based on the rate at which they disappear in tame rabbits killed experimentally (Brambell, Henderson & Mills, 1948). Applying the theoretical distribution defined on p. 394 to the observed distribution in the 11- to 15-day age group it can be calculated that 56% of the 12-day-old litters appear to be in process of being lost. This method gives an unduly high value as compared to the others, but it may be concluded with some confidence that the true proportion of litters lost after implantation is not less than 35% although it may be considerably higher.

The loss of embryos as units after implantation in litters that survive is difficult to estimate with the precision that is possible in the case of loss before implantation because it is small in comparison to the loss of whole litters, which masks it. The theoretical distribution of best fit for the whole sample of data of all stages is given by equation (1) when $p = 0{\cdot}0164$. This agrees well with the proportion of embryos lost of 0·0116 in the 26 days and over age group of litters approaching full term. Calculating the value of p for successive 5-day age groups, it is found to rise consistently to 0·035 in the 16- to 20-day group. If this maximum estimate is accepted then the subsequent decline to the 0·0116 observed in the latest age group must be due to the omission from the counts of late stages of some placental sites from which the embryos had disappeared early. It is probable that the loss of embryos as units after implantation in litters that survive is between 1 and 3·5 % of those which became implanted in these litters.

The proportion of litters lost as units after implantation is inversely related to the body weight of the mother, as it is to the size of litter at implantation. This is to be expected, since size of litter is directly related to body weight. It varies also both from one locality to another in Great Britain and from one year to another in the same locality. The incidence of the total mortality after implantation is not closely correlated with the state of mammary activity of the mothers but the loss of whole litters at 11–15 days pregnant does appear to be heavier in lactating animals.

The results of this analysis of the distribution of the prenatal mortality after implantation in wild rabbits may help to elucidate the factors which contribute to the death of embryos. Since the loss of embryos in surviving litters approaches a random distribution it is probable that a large number of separate factors must contribute. Presumably these are mainly intrinsic, since they operate on the embryos as units and not on the litters. The loss of whole litters, on the other hand, is almost certainly due to extrinsic factors affecting the maternal environment. It was considered at first that the death and autolysis of one of the embryos, from intrinsic causes, might so adversely affect the uterine environment of the others as to bring about their death. Thus the death of the first embryo might result from any one of many factors each responsible for only a small part of the loss of embryos as units, and which in sum result in an almost random distribution of the loss. Hence the litters suffering this loss would tend to be distributed as $1 - q^x$, where x is the size of litter. Since the loss of the whole litter would result from the death of one, the proportion of litters lost would increase geometrically with the size of litter, whereas it has been shown that in fact it decreases. The most striking characteristic of the loss of whole litters is that the greater part of it occurs in a brief and clearly defined period following implantation, and hence it is probable that this uniformity results from the same cause being responsible for the mortality in the majority of cases. Both because of its characteristic uniformity and because it is much the largest fraction of the total loss the problem of its cause is of particular interest. Since it is distributed independently of the loss before implantation it may be deduced that it is due to factors which do not operate to the disadvantage of the embryos to any appreciable extent until they become attached to the uterus. The mortality must be due to factors operating through the mother.

Hammond (1928) records an inbred strain of rabbits in which much foetal atrophy occurred and he was able to show that, in this instance, it was due to a recessive Mendelian factor, possibly a multiple factor, which operated through the mother on the embryos in the uterus. Much of the mortality was spread over later stages of development than that observed in the wild rabbits. The loss in wild rabbits cannot be accounted for in this way since it occurs in suckling mothers, which, therefore, must have borne a living litter previously.

The facts that (*a*) the loss takes place at a stage of development when the nutritive requirements of the embryo can be only a small fraction of those in late pregnancy; (*b*) that the incidence of the loss is less in large litters, which require more nutriment; and (*c*) that loss occurs, although the incidence appears to be less, in non-suckling as well as in suckling animals, despite the fact that lactation is a much greater drain upon the mother even than late pregnancy, would seem to preclude a simple quantitative nutritional deficiency of the mothers as the cause. The decline in the proportion of litters lost both with increasing size of litter at implantation and with increasing body size of the mother is significant. It might have been expected that the larger the litter the harder it would be for the mother to support it, but it appears that this is not a determining factor. The results might be interpreted instead as indicating that the same factors which favour large body size and a large number of embryos implanting favour the survival of the litter. Wild rabbits differ notably from tame rabbits in that they normally maintain pregnancy while they are suckling. Hammond (1925) says of the tame varieties: 'While it is the rule in the rabbit that pregnancy does not occur during lactation there are two circumstances under which it is possible: (*a*) where only a small number of young are being suckled, and (*b*) in times of abundant nutrition suitable for breeding.' Clearly he is using 'pregnancy' as synonymous with containing implanted embryos and he attributes the failure to inadequate nutrition for he says 'the lack of uterine nutrition as an effect of suckling causes the death of the blastocyst and its absorption'. Vitamins or trace elements suggest themselves as possible qualitative deficiencies. Both the wide geographical distribution of the mortality and its occurrence at all seasons of the year render a close correlation with either soil characters or food plants unlikely.

One other line of evidence as to the possible cause of the mortality exists (Brambell & Mills, 1947*a*). No consistently occurring major abnormalities capable of causing death have been recognized in the dead embryos themselves, but a common abnormality has been observed at earlier stages which might be responsible for the subsequent death of the embryos, to which there is evidence that it is related. The yolk-sacs of many embryos of wild rabbits at stages around 8 and 9 days post-coitum contained gelatinous clots and in many such cases the successive embryos in the uterus were connected by strands of similar material traversing the uterine lumen. Histological examination suggested that the clot was formed of fibrin. Experimental investigation of the fluid yolk-sac contents of normal 9-day embryos of tame rabbits enabled the presence of considerable quantities of fibrinogen, which clotted when thrombin was added to the citrated fluid, to be demonstrated. Ultracentrifuge and electrophoretic analyses of the yolk-sac fluid (Brambell & Hemmings, with McCarthy and Kekwick, 1948) showed that all the plasma proteins, albumin, α-, β- and γ-globulin, and fibrinogen were present and in similar relative proportions to those in the blood. Finally, it has been possible to show immunologically, that the agglutinin to *Brucella abortus*, whether actively or passively acquired by the mother, and, if passively, whether of rabbit or bovine origin, passes freely from the maternal circulation into the yolk-sac cavity of the embryos during the 7th and 8th days of pregnancy (Brambell, Hemmings & Rowlands, 1948). It may be concluded that the bilaminar omphalopleur of the yolk-sac wall of the rabbit embryo at these stages allows the free passage of maternal proteins as such, without molecular changes that could be detected immunologically, into the yolk-sac cavity and that the clots observed in wild rabbit embryos were formed from maternal fibrinogen. The factors which result in clotting, and the mechanism by which it is effected, are unknown, and it is not established that the clotting is causally related to the mortality, but the results are suggestive.

VIII. THE TOTAL LOSS BEFORE BIRTH

To arrive at an estimate of the total loss of ova between ovulation and full term in wild rabbits it is necessary to convert the estimates of loss after implantation which have been given as percentages of implanted embryos, into terms of ova ovulated. Taking the minimum estimates in all cases we find that 35·7 % of the litters are lost entirely, of which 1·0 % are lost before implantation and 34·7 % after implantation, and that 7·6 % of the ova are lost in the surviving litters, of which 7·0 % are lost before implantation and 0·6 % after implantation. The observed loss, both before and after implantation combined, in the 26 days and over age group is 11·35 % which, assuming that 64·3 % of the litters have survived to compose this age group, is 7·3 % of the ova ovulated in all litters, which agrees well with the estimate. Thus the total loss of ova ovulated is 43·3 % on the lowest estimate of which 10·2 % is lost before implantation and most of the remainder before mid-pregnancy. Yet, without critical analysis, a simple comparison of the counts of corpora lutea and surviving embryos in the whole sample of pregnancies revealed a discrepancy of only 16·0 %, a value that can now be seen to be far too small and quite valueless as an estimate of the prenatal mortality experienced.

This estimate of prenatal mortality in wild rabbits does not include any loss that may occur at the time of parturition. It is obviously impracticable to obtain information as to the proportion of stillbirths in a population of wild animals. Such information is very difficult to obtain even for laboratory animals. Feldman (1926) estimated that the percentage of abortions and stillbirths in Norway rats was 7·9 %, and King (1921) estimated the percentage of stillbirths, excluding abortions, in these animals at 1·3 %. Ransom (1941) estimated the loss during the second half of gestation in a laboratory colony of field voles at 21·1%, from counts of embryos at mid-term made by palpation and of the newborn young. Heape (1899) estimates the percentage of abortions in sheep at 2·3 from data comprising records of 97,239 ewes from 350 flocks. The literature on abortion and stillbirth in man is extensive and scarcely relevant. Data of abortion in man are so complicated by the prevalence of induced abortion, criminal or otherwise, that they are practically worthless for comparative purposes. Neither is the percentage of stillbirths in civilized countries of much comparative significance, in view of the problematic effects of medical interference, but a number of estimates varying from 3·0 to 5·8% are quoted by Parkes (1926*b*).

IX. THE REMOVAL OF DEAD EMBRYOS

Dead embryos and their membranes are removed from the uterus either by being forcibly expelled relatively intact or by being gradually autolysed or mummified *in situ*. The former process is called abortion and the latter reabsorption or resorption. Both processes occur in most, if not all, mammals, but whereas abortion is the commoner in monotocous species, reabsorption is the rule in polytocous forms. Since abortion involves the abrupt termination of pregnancy whereas reabsorption does not, it being quite frequent for neighbouring embryos to one which is dead to continue to develop and to survive to term, the latter should be of survival value to mammals which produce several young at a birth. The significance of the two processes in relation to studies on sterility and prenatal mortality is different.

Abortion is a muscular process, analogous to parturition, which, if carried to completion, results in the expulsion of the entire conceptus or conceptuses from the tract. It is probable that it results from the premature release of the same physiological mechanisms which normally result in parturition, and consequently it will involve the relaxation of the inhibition which prevents the occurrence of labour during gestation. Parkes & Bellerby (1926), Parkes (1930) and Marrian & Newton (1935) produced abortion in mice by administration of natural oestrogen, followed in the last two mentioned cases, by oxytocin. Abortion may result in the loss of some embryos actually surviving at the time of the event or it may occur very shortly after the death of the embryos. Moreover, a uterus which has aborted is difficult or, it may be, impossible to distinguish from a post-partum uterus. The possibility of detecting prenatal mortality resulting in or from abortion at autopsy is slight. Comparative histological study of post-abortion and post-partum uteri with a view to establishing distinguishing characteristics is desirable. We are aware of only one instance where it was possible to establish on purely histological grounds that abortion, not parturition, had occurred in a small mammal. Brambell & Davies (1941) described the uteri of a multimammate mouse with unhealed placental sites on the antimesometrial side, which must have been due to abortion occurring soon after implantation, which is antimesometrial, and before the establishment of the placenta, which is mesometrial. Abortuses can, however, be recovered and detected in the intact animal, whereas reabsorption is almost impossible to detect except at autopsy. The habit of many mammals of immediately eating the abortuses, as they do the afterbirth at parturition, renders the detection of abortion uncertain.

The process of reabsorption is a gradual one and its duration and result depend on the stage of development attained by the embryo at the time of death as well as upon other factors. Early embryos are rapidly and completely disintegrated, whereas late embryos may resist complete maceration and their remains, becoming shrivelled and mummified, may persist till full term. A dead embryo rapidly becomes flaccid and, in consequence, distorted in shape. The head especially, in embryos which die before the cartilaginous skeleton is developed, loses shape rapidly and the cervical flexure is lost. Early embryos become white and opaque, but discoloration occurs in later embryos. The viscera disintegrate first and the limbs remain recognizable even after the rest of the embryo has fragmented. In later embryos the macerated skeleton may persist and mummification may be favoured when death occurs after cuticularization of the skin. The embryonic fluids escape from the dead membranes, which collapse and closely invest the embryonic remains. These changes are characteristic of embryos dying during the life of the mother, and it is surprisingly easy to distinguish at autopsy between such embryos and those which survived to the time of the mother's death, even in uteri in which post-mortem changes are advanced.

The dissolution of the embryos has all the appearance of aseptic autolysis. The experiments of Long & Parkes (1924) which demonstrated the autolysis of embryos *in vitro* incubated under aseptic conditions, confirm this interpretation. The soluble products of autolysis probably drain away through the cervix and are discharged. Late reabsorption may result in a characteristic dark-brown vaginal discharge, and even in the expulsion of recognizable embryonic or placental remains at full term, which may be detected in the living animal by close observation. There is no evidence that any of the products of embryonic disintegration are absorbed by the maternal tissues but the possibility has not been excluded. Kerr (1947) killed the embryos and embryonic membranes in mice at 6- and 10-day stages by subcutaneous injection of colchicine. The embryonic tissues died within a few hours of injection and a gradual disintegration by autolysis and spreading necrosis followed. This was accompanied by the passage of large numbers of leucocytes into the lumen. No evidence was obtained of the passage of any of these leucocytes back into the maternal tissues, but the possibility could not be excluded. Oestrus recurred about 6 days after injection and the products of embryonic disintegration were flushed out of the tract with the fluid which distends the murine uterus at oestrus. The work of Kerr & Robertson (1943, 1947) on cattle, however, suggest that the uterine mucosa is absorptive and show that immunological responses are induced by the injection of antigens into the uterine lumen.

It is well known that the death of the embryo does not necessarily involve the death of the embryonic membranes. These may grow and even survive to full term. Survival of the embryonic membranes, especially the trophoblast, after natural death of the embryo, has been described in several mammals (Meyer, 1917) and frequently in man (Mall & Meyer, 1921; Hertig & Edmonds, 1940). More valuable information has been obtained by the experimental induction of embryonic death at known stages of pregnancy. Various methods have been employed, such as the administration of natural oestrogens (Parkes & Bellerby, 1926; Smith, 1926; Parkes, 1930; Newton, 1935; Huggett & Pritchard, 1945) or synthetic oestrogens (Parkes, Dodds & Noble, 1938; Brambell *et al.* 1948), colchicine (Kerr, 1947), gonadotropins (Engle & Mermod, 1928; Huggett & Pritchard, 1945) vitamin E deficiency (Evans & Burr, 1927; Urner, 1931) and mechanical interference (Giacomini, 1892; Hammond, 1917; Newton, 1935; Courrier & Gros, 1936; van Wagenen & Newton, 1943; Huggett & Pritchard, 1945; Pritchard & Huggett, 1947; Brambell *et al.* 1948). Administration of oestrogens or colchicine appears to result in the death of the membranes as well as the embryos, whereas the membranes may survive the destruction of the embryo by vitamin E deficiency or mechanical interference. Giacomini (1892) found that the membranes continued to grow for several days after the operative removal of rabbit embryos, before the placenta was fully developed. Hammond (1917) also found that the placentae sometimes survived the removal of the embryos in rabbits. Newton (1935) destroyed all the embryos without dislodging the placentae in pregnant mice by manipulation under anaesthesia on the 12th–15th days. He found that the placentae survived and were delivered at normal full term. Evans & Burr (1927) found that the embryos died, usually about the 13th day, in pregnant rats suffering from vitamin E deficiency but that the trophoblast continued to grow until the 16th day. Pritchard & Huggett (1947) killed all the embryos in pregnant rats by surgical interference at 10- to 19-day stages. The embryo, amnion and yolk-sac splanchnopleur disintegrated, but the placental trophoblast, allantois and yolk-sac omphalopleur survived. The surviving membranes grew rapidly until the 16th day and histological differentiation was normal except for the absence of foetal vessels. The placentae were expelled at full term. Brambell *et al.* (1948) killed all the embryos in pregnant rabbits by a single massive dose of stilboestrol administered by subcutaneous injection in each case. The embryos and membranes died within 24 hr. of injection. Reabsorption invariably occurred after death at 11½ or 16 days post-coitum, the embryos disintegrating within 3–3½ or 4–4½ days respectively and the reabsorption sites remaining recognizable as such and distinguishable from post-partum placental sites for 9–10 days. Abortion occurred when the embryos were killed at 20 days. When all the embryos were killed by surgical interference at 16 or 20 days, abortion followed in most cases, though only after an initial period of autolysis in the former. Reabsorption, when it did occur, proceeded much more slowly than after stilboestrol and fragmentation did not occur in less than 7 days after death. Reabsorption, not abortion, was the rule when some only of the embryos were killed surgically and others survived. Sometimes the dead embryos were still intact, though shrivelled and deformed, at full term. These differences were attributed to the death of the membranes after stilboestrol treatment and their survival after surgical interference. Abortion was not observed in any of the experiments before the 19th day. Probably it cannot occur until a zone of weakness has developed, which appears at this stage and facilitates the separation of the placenta from the uterine wall.

Much more work on a variety of species is required before we can hope to understand the factors which determine the time relations and the method of removal of dead embryos. It is possible, however, to draw some tentative conclusions from the available information, reviewed above. Abortion, defined as the expulsion of the residue of a conceptus dying before full term, and reabsorption, defined as the gradual disintegration *in situ* of a dead conceptus, should not be regarded as distinct and contrasted processes, but rather as parts of a single process. Thus an interval, however brief, during which autolysis will proceed, must supervene between the death of an embryo and its abortion and conversely, however far autolysis proceeds *in situ*, it appears that the products are ultimately expelled from the tract, at full term, if not before. In this sense reabsorption is the invariable prelude to abortion and abortion the termination of every reabsorption. Nevertheless, the duration of the reabsorptive phase varies widely not only from species to species, but also according to the stage at which foetal death occurs, and whether or not the membranes survive or other living conceptuses are present in the uterus. The death of all the embryos and membranes results in the premature termination of pregnancy. Whether the products simply drain out of the tract or are flushed out, as in murines, or whether they are actively expelled by a muscular process resembling labour may depend simply on their degree of solidity. The survival of some embryos, or even of foetal placental tissues only, may be sufficient to maintain the physiological state of gestation and hence result in the expulsion of the conceptuses only at full term. The duration of the phase of reabsorption would then depend only on the stage of gestation at which death occurred. This interpretation conforms with the frequent occurrence of abortion in monotocous species and of reabsorption in polytocous species.

X. CONCLUSIONS

It remains to draw together in this concluding section the more important results of these studies on wild rabbits and to consider their general implications in relation to the problem of prenatal mortality in mammals generally.

The fact of greatest significance which emerges from analysis of the rabbit data is that the total prenatal mortality is much heavier than could have been supposed either from information previously available or from uncritical examination of the material. The investigation cannot be considered completely satisfactory in so far as the nature of the data and the limitations of the technique did not permit of distinguishing between loss of unfertilized ova and subsequent death of developing embryos, or of determining the mortality of full term foetuses occurring at the time of parturition. Yet it is clear that the loss between ovulation and full term is not less than 43 % of ova ovulated, and may be, and indeed probably is, substantially heavier. This is an enormous wastage, and, if it can be taken as any guide to the possible incidence of prenatal mortality in other species, it provides an impressive measure of the increased fertility which could result from diminution of prenatal mortality.

Scarcely less significant is the revelation of the extent to which estimates of prenatal mortality based on inadequate methods of analysis can be misleading. A convincing example is provided by the rabbit data for a simple comparison of the numbers of corpora lutea and of surviving embryos in the whole sample of post-implantation stages shows a loss of only 16%, which bears no relation to the true loss of not less than 43%, yet this is the method which has been commonly used to provide a measure of the prenatal mortality when the data are derived from ovaries and pregnant uteri, as with samples of wild populations or slaughterhouse material. Indeed it is difficult to find in the existing literature estimates of the total prenatal mortality in any species on which reliance can be placed, but it is unnecessary to labour the point of the inadequacy of existing knowledge, which emerges clearly from this review.

Another significant conclusion is that the greater part of the prenatal mortality in wild rabbits occurs at early stages of pregnancy, before, during and immediately after implantation. The mortality reaches a maximum by the 12th day post-coitum and comparatively little loss occurs after the 15th day. Pig and human embryos attain corresponding stages of development to that of the 15-day rabbit embryo at approximately 4 and 5 weeks after ovulation respectively. The difficulty of detecting in living animals prenatal mortality occurring at such early stages of development can be appreciated when it is realized that in the rabbit it need not result in any prolongation of pseudopregnancy, in the pig it need only prolong the recurrence of oestrus by 1 week and in man it would involve only one missed menstrual period. If the mortality is concentrated at corresponding stages of development in many mammals, as such information as is available suggests, then it is clear that future studies should be directed especially to these early stages.

It has been shown that analysis of the distribution of the prenatal mortality, at least in polytocous animals such as the rabbit, can yield valuable information as to the proportions of the mortality which are to be attributed to maternal and to foetal failure respectively. Moreover, in wild rabbits the greater part of the loss which occurs at, and shortly after, the time of implantation would appear to be due to maternal causes, rather than to genetic deficiency of the embryo. Since maternal factors seem to be the more accessible to therapeutic treatment, and, if overcome, to hold no threat to the future viability of the embryo, as would genetic deficiency on their part, the prospect of artificially increasing fertility by decreasing the loss of early embryos appears correspondingly brighter.

The subject of prenatal mortality in mammals is one of both great theoretical and practical significance yet it has received less attention than almost any other aspect of mammalian reproduction. Bearing, as it does, on the one hand on the oestrous cycle and on the other on the embryology and placentation of mammals, it cannot fail to derive great benefit from the notable advances of recent years in these fields, and it may be that it will have much to contribute to the ordering and understanding of the extraordinary diversity of these phenomena displayed by representatives of different orders, and even of different families and genera, of mammals. It is fundamental to problems of fertility and fluctuation in numbers of wild mammals. As such it is of practical importance in relation to the control of mammalian pests and vectors. It is also fundamental to the study of sterility in man and the domestic mammals, and hence the urgency of the problem. The diversity of reproductive phenomena in mammals, referred to above, warrants the expectation of a corresponding diversity of prenatal mortality. It is improbable that the incidence and distribution of prenatal mortality in one species will resemble that in others, but the technique and method of analysis employed can be adapted and applied to all. A thorough knowledge of the oestrous cycle and embryology of the species is indispensable in planning research on prenatal mortality on sound lines and substantial practical experience of the material is essential for its conduct.

XI. SUMMARY

1. The intention of this article is to provide a critical examination of the methods of estimating the amount, and of analysing the distribution, of prenatal mortality in mammals, in the light of researches on wild rabbits.

2. The reasons why the rabbit provides particularly favourable material for such studies are considered. A summary of the oestrous cycle and embryology provides an essential basis for study of the prenatal mortality.

3. The size of litter at conception is limited by the number of ova ovulated at oestrus, which varies from species to species. The number ovulated at the preceding oestrus in any individual can be estimated from the number of corpora lutea in the ovaries. The magnitudes of, and factors responsible for, errors in such estimates are examined. The frequency distribution and the relation to body weight of the number of ova ovulated are examined. Transmigration of ova from one ovary to the uterus of the opposite side may occur.

4. Several indirect methods of estimating the total prenatal mortality are available, and both the conditions under which each is applicable and the limitations of the results which each can yield are analysed.

5. Estimates based on counts made at autopsy of corpora lutea and of surviving embryos are of little value unless counts of implantation sites are included. It is then possible to separate data of loss before implantation from those of loss after implantation. The nature and limitations of these two sets of data are different.

6. The data available in the literature of loss before implantation in various species are considered. The loss of ova before implantation in wild rabbits is between 10·2 and 13·0% of those ovulated, of which between 1·0 and 3·6% are lost in litters in which none of the ova survive to become implanted. The distribution of the loss and its significance are examined.

7. The data available in the literature of loss after implantation in various species are summarized. It is shown that estimates based on such data may be very misleading. Analysis of the loss after implantation in wild rabbits shows that not less than 35% of the litters surviving implantation are lost *in toto*, mainly between the 11th and 15th days post-coitum. Between 1·0 and 3·5% of the embryos which have implanted are lost in the surviving litters. The distribution of the mortality, which is complex, is analysed and the

bearing of the statistical, histological and experimental results obtained on the problem of the causes of the loss is discussed.

8. The total loss of ova ovulated in wild rabbits both before and after implantation combined, but excluding loss at parturition, is 43·3% on the lowest estimate, of which total 10·2% are lost before implantation and most of the remainder before mid-term. The loss of ova in litters that do not survive is 35·7%, and in litters that do survive is 7·6%.

9. The method of removal of dead embryos from the uterus is described and the processes of reabsorption and abortion are compared. The significance of these processes in relation to studies on prenatal mortality is discussed.

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THE INSECT CUTICLE

By V. B. WIGGLESWORTH
Department of Zoology, University of Cambridge

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The rapid extension of knowledge of the arthropod cuticle which has taken place during the past ten years has emphasized the complexity of this remarkable structure, and has, indeed, so transformed our views that a general survey, in which recent advances may be set against the background of earlier work, seems called for. The present review is limited to the insects, save where passing reference to other arthropods may serve to illuminate the theme.

I. INTRODUCTION: STRUCTURE AND FUNCTIONAL ADAPTATIONS OF THE CUTICLE

1. *Basement membrane*

It was first recognized by C. Schmidt (1845) that the matrix of the arthropod cuticle is a single layer of epidermal cells, the 'chitinogenic cells' of Haeckel (1857), often called the 'hypodermis' (Fig. 1). These cells rest upon a basement membrane, the origin and nature of which is uncertain. In common with the sarcolemma, the membrane around the fat body, and other basement membranes, it can be impregnated with silver (Ochsé, 1946). Some authors favour the view that it is a product of the epidermal cells: in the queen termite, for example, Ahrens (1930) describes branching processes ('intracellular apodemes') extending from the membrane into the cells. Others consider it to be in the nature of connective tissue, derived from the transformed cytoplasm of phagocytic blood cells (Lazarenko, 1925).

The stellate cells which often lie below the epidermis were regarded by Mayer (1896) as secreting the basement membrane as an intercellular substance filling the lacunae between them. Certainly it is easy to imagine that the flattened, stellate haemocytes which apply themselves to the membrane may be contributing to its substance (Wigglesworth, 1933). During the healing of wounds, however, it is possible to follow step by step the formation of the new basement membrane. It clearly arises by the condensation of membranes derived from interlacing cells which ultimately degenerate; but the cells in question appear to come from the epidermis (Wigglesworth, 1937). The same conclusion was reached by Nowikoff (1905) in the phyllopod *Limnadia*.

2. *Epidermis*

In the mature insect, or in the young stages when they are not in the process of moulting, the epidermis is exceedingly attenuated. It is seen in its full development only when the cuticle is being laid down, and then is far more conspicuous where the cuticle is thick (Wigglesworth, 1948). The apical region of the epidermal cell is usually striated, the striae being continuous with the vertical filaments of the cuticle (Nordenskiöld, 1908; Kuhnelt, 1928*c*). It seems probable that these filaments extend far into the body of the cell, perhaps to the basement membrane, for when the epidermal cells contain granules of pigment these may arrange themselves in vertical lines (Wigglesworth, 1933).

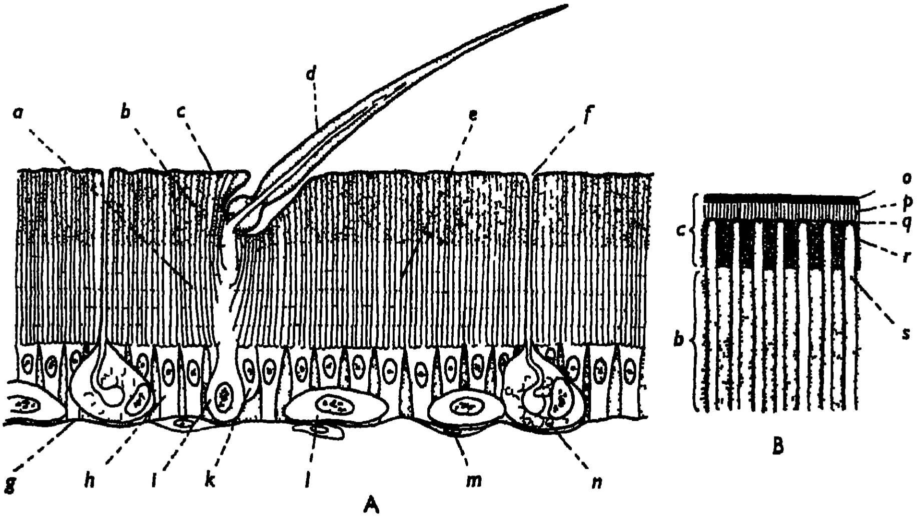


Fig. 1. A, ideal section of the insect integument; B, schematic section of the epicuticle. *a*, laminated endocuticle; *b*, exocuticle; *c*, epicuticle; *d*, bristle; *e*, pore canals; *f*, duct of dermal gland; *g*, basement membrane; *h*, epidermal (hypodermal) cell; *i*, trichogen cell; *k*, tormogen cell; *l*, oenocyte; *m*, haemocyte adherent to basement membrane; *n*, dermal gland; *o*, cement layer of epicuticle; *p*, wax layer; *q*, 'polyphenol layer'; *r*, cuticulin layer; *s*, pore canal.

Differentiated from the epidermis during development are the dermal glands, which send fine ducts through the cuticle, and the oenocytes. In some insects the oenocytes become entirely separated from the epidermis, but often they remain applied to its lower surface, adding greatly to its thickness (Minchin, 1888; Mingazzini, 1889; Wigglesworth, 1933). Glands and oenocytes will be considered further when the deposition of the cuticle is discussed (p. 434).

3. *Layers of the cuticle*

The arthropod cuticle consists of an outer, harder and more resistant layer, with an outermost limiting membrane, insoluble in cold concentrated hydrochloric and sulphuric acids, and an inner softer and more soluble layer. The most convenient terminology for these layers is that proposed by Campbell (1929) who refers to them as 'epicuticle', 'exocuticle' and 'endocuticle'. Other terms which have been used are as follows:

Epicuticle: Grenzhäutchen (Bütschli, 1898; Kapzov, 1911); Grenzsaum (Hass, 1916); Grenzlamelle (Kühnelt, 1928*c*).

Exocuticle: Aussenlage (Bütschli, 1898); Emailschicht (Biedermann, 1903); Lackschicht (Schulze, 1913); Pigmentschicht (Hass, 1916; Kremer, 1920); 'Epidermis' (Berlese, 1909); Primary cuticula (Tower, 1906).

Endocuticle: Innenlage (Bütschli, 1898; Kapzov, 1911); Hauptlage (Hass, 1916, etc.); 'Dermis' (Berlese, 1909); Secondary cuticula (Tower, 1906).

In the elytra of some beetles a layer with vertical rods (Alveolarsaum of Bütschli, Stäbchenschicht of Biedermann) is interposed below the epicuticle.

The line between exocuticle and endocuticle represents the boundary between the cuticle formed before and after moulting. Often the outer part remains soft, and then the exocuticle is said to be wanting. For these reasons it has been suggested that the cuticle be regarded as made up of two primary layers, epicuticle and endocuticle, the outer part of the latter being sometimes hardened to form an exocuticle (Wigglesworth, 1933). This terminology was applied to the Crustacea by Drach (1939) who refers to the pre-exuvial endocuticle (sometimes hardened and darkened) and the post-exuvial endocuticle which always remains soft.

In general the endocuticle is colourless and flexible, the exocuticle hard, brittle and pigmented—sometimes a pale amber, sometimes a dark reddish brown. The epicuticle may be colourless, amber or dark grey. The exocuticle commonly makes up about one-third of the total thickness; but it may amount to no more than about one-tenth, as in the larva of *Tenebrio* (Plotnikov, 1904). The epicuticle is at most only a few microns thick.

The total thickness of the cuticle varies greatly in different insects. The following are a few values from the literature:

Periplaneta adult: 40μ (Richards & Anderson, 1942); *Bombyx mori* larva in final instar: 10μ increasing to 40μ (Kuwana, 1933); *Culex* larva, final instar: 0·75–2μ (Richards & Anderson, 1942); *Sarcophaga* larva, final instar: 10μ increasing to 240μ (Dennell, 1946); *Rhodnius*, abdominal tergites of adult: 30μ; abdominal tergites of 5th instar nymph: 60μ (Wigglesworth, 1933); *Tenebrio* adult, abdominal tergites: 4μ; sternites: 36μ (Wigglesworth, 1948); *Dytiscus* larva: up to 370μ; pupa: 30–40μ (Korschelt, 1923).

4. *Epicuticle*

In recent years it has been shown that the 'epicuticle' is a complex structure made up of several layers. These are not readily differentiated histologically; their existence has been deduced by other means which will be discussed when the permeability (p. 426) and the deposition (p. 434) of the cuticle are described; but a few histological observations may be noted here.

The epicuticle is usually described as being less than 1μ thick. In the mosquito larva, as measured with the electron microscope, it is only 0·03μ (Richards &

Anderson, 1942). But it may be far thicker: 2μ in *Periplaneta* (Richards & Anderson, 1942); 2–4μ in certain parts of the Mallophagan *Eomenacanthus* (Webb, 1947); 4μ in the larva of *Sarcophaga* (Dennell, 1946). Richards & Anderson (1942) recognized two layers in the epicuticle of the cockroach. Immersed in concentrated nitric acid at 60–70° C. it separated into two sheets, an outer epicuticle, very thin (0·02–0·03μ) and colourless, and a thicker amber-coloured inner epicuticle (about 2μ). The abrupt ending of the pore canals 2μ below the outer surface was the only morphological separation seen in the electron microscope between exo- and epicuticle.

Dennell (1946) likewise recognizes an epicuticle of two layers in the larva of *Sarcophaga*: an outer epicuticle 1μ thick and an inner epicuticle of 4μ. In the hardened puparium of *Sarcophaga* (and in the sclerites of *Tenebrio*) the inner epicuticle becomes indistinguishable histologically from the exocuticle. In the thick epicuticle of *Eomenacanthus*, Webb (1947) describes three layers with different staining reactions, all penetrated by the pore canals, the whole being covered by a layer of 'cement'.

In *Rhodnius* (Wigglesworth, 1945, 1947*a*) and *Tenebrio* (Wigglesworth, 1948), in which the deposition of the epicuticle has been followed in detail (p. 435), there is a layer of indeterminate thickness, the 'cuticulin' layer, which is the refractile 'epicuticle' as seen in histological sections, and over the surface of this, separable by treatment with wax solvents, there is a very thin colourless layer, the 'cement' layer. A similar structure is described in the tick *Ornithodorus* (Lees, 1947) and in the caterpillar of *Diataraxia* (Way, 1948).

5. *Endocuticle and exocuticle*

The endocuticle is usually made up of obvious horizontal lamellae which increase in thickness towards the inner surface. These are commonly regarded as providing for flexibility and for stretching by sliding over one another (Ahrens, 1930). Such movements take place particularly at the intersegmental membranes. In these regions the cuticle may be thinner than elsewhere (10μ as compared with 40μ on the sternites of *Tenebrio* (Wigglesworth, 1948)); often it is somewhat thicker (50μ as compared with 30μ in the tergites of the *Rhodnius* adult (Wigglesworth, 1933)); but an exocuticle is wanting or, as in *Tenebrio* larva (Plotnikov, 1904) and *Liogryllus* (Hass, 1916), it is broken up into little wedge-shaped blocks or into elongated rod-like thickenings, as in *Meloë* (Escherich, 1897).

Where great distension has to be provided for, as in the pleural membrane of blood-sucking insects, or the general surface of the abdomen in the *Rhodnius* nymph (Wigglesworth, 1933) or in female ticks (Lees, 1946), an exocuticle is absent and the epicuticle in the unstretched state is thrown into deep folds.

Rigidity is commonly supplied by the hardness of the exocuticle; but in the Coleoptera the endocuticle has a characteristic structure which perhaps contributes to the firmness of the cuticle. As was first described by Meyer (1842) in the elytra of the stag beetle *Lucanus*, the endocuticle is made up of layers composed of clear colourless rods running parallel but often anastomosing. The rods in successive layers run at an angle of 45° or 90° to those above or below, so that where they cross there are star-like figures with eight rays. The details of this structure have often been described (Beauregard, 1885; Berlese, 1909; Kapzov, 1911; Stegemann, 1929, 1930). The angle between the rods in successive layers is commonly stated to be 60° (Korschelt, 1923) and to be correlated with the hexagonal pattern of the epidermal cells (Langner, 1937). The layers of parallel strands have been termed 'Balkenlagen'. In cross-section the strands are speckled like tendons and are therefore believed to consist of bundles of fibrils, bundles which are compressed with their sharp edge inwards (Biedermann, 1902, 1903). As Biedermann (1903) points out, fibrillar structures with fibres running in different directions in successive layers are common in the skeletal structures of animals, such as the placoid scales of Selachians or the cornea of the eye.

6. *Pore canals*

Leydig (1855) confirmed Meyer's observations on the structure of the elytra of beetles and described, in addition, fine processes running vertically from the cells through the lamellae of the cuticle. These processes had been observed in *Astacus* by Valentin (1837); they were believed by Leydig to contain a nutritive fluid; he called them 'pore canals'. They are often invisible in histological sections of the endocuticle (though the exocuticle generally shows a distinct vertical striation (Ahrens, 1930)), and some authors have therefore doubted their existence. But they are readily seen in both endocuticle and exocuticle in fresh sections cut with the freezing microtome and examined in water. They have been described, among others, by Braun (1875), Tullberg (1881), Vitzou (1882) and Hass (1916) in Crustacea, Langner (1937) in Diplopoda, Nordenskiold (1908) and Lees (1946) in arachnids, and among insects by Bütschli (1894), Biedermann (1903), Kapzov (1911) in beetles, Holmgren (1902*a*, *b*), Plotnikov (1904) and Berlese (1909) in various insects, Wigglesworth (1933, 1948) in *Rhodnius*, *Tenebrio*, *Periplaneta*, Richards & Anderson (1942) in *Periplaneta*, Dennell (1946) in *Sarcophaga* larva and Webb (1947) in *Eomenacanthus*. Pore canals are said to be absent from the cuticle of the mosquito larva as studied with the electron microscope (Richards & Anderson, 1942); and they are absent from the inner endocuticle of the *Sarcophaga* larva, which is laid down shortly before the formation of the puparium (Dennell, 1946).

The pore canals certainly exist; but there is still some doubt about their extent and their function. They certainly do not open to the exterior; they are usually said to end below the epicuticle—but that will be best discussed when the deposition of the outermost layers is described (p. 435). At the inner end they reach the cells and, at least in the recently formed cuticle, cytoplasmic processes can be seen running from the epidermal cells into the canals (*Leptinotarsa*, Tower, 1906; *Tenebrio*, Wigglesworth, 1948; *Sarcophaga* larva, Dennell, 1946).

If the cuticle is examined fresh in surface view the pore canals appear as minute points, usually grouped in polygonal areas corresponding to the cells, with clear boundaries between. On focusing the microscope up and down they appear to rotate, indicating that they run a spiral course (Vitzou (1882) in Crustacea; Plotnikov (1904) and Kapzov (1911) in the head capsule of *Oryctes*; Hass (1916) in *Gryllotalpa*, etc.; Wigglesworth (1933) in *Rhodnius*). In the larva of *Sarcophaga* (Dennell, 1946) they are relatively coarse structures, 1·0μ in diameter, 50–70 arising from each of the large epidermal cells, about 15,000 canals per sq.mm. In this larva, their innermost section is straight, they have an intermediate section roughly coiled in a spiral with a pitch of 2·5μ, and a superficial branched section where they show the tufted form described by Plotnikov (1904) in larvae of Syrphidae and in the silkworm and by Nordenskiöld (1908) and Lees (1946) in the tick.

In the cockroach they are much finer structures (Richards & Anderson, 1942). What appear under the light microscope as straight parallel threads 1μ in diameter are seen with the electron microscope to consist of hollow tubes with an average diameter of 0·15μ thrown into a tight helix with a pitch of 0·25μ throughout most of their course, with a straight section about 0·4μ in diameter near the epidermal cells. It is estimated that if they were drawn out their total length would be about twice the thickness of the cuticle. There are about 200 arising from each of the comparatively small epidermal cells, some 1,200,000 per sq.mm.; they make up perhaps 5–6% of the total volume of the cuticle.

Deegener (1911) went so far as to consider the pore canals as no more than vertical striations, the manifestation of the intracellular framework still remaining visible after the transformation of the cell substance into cuticle. Holmgren (1902*b*) and Plotnikov (1904) also regarded them as vertical fibres which had suffered the same conversion into skeletal substance as the remainder of the cuticle. There is no doubt that this transformation does occur in some species. Braun (1875) described hair-like processes, drawn out from the pore canals, which are visible when the innermost layer of the cuticle in *Astacus* is pulled away. Biedermann (1903) observed the same thing in the elytra of *Lucanus* and in certain Crustacea, and so did Hass (1916). It has been demonstrated very convincingly by Dennell (1946) in the mature larva of *Sarcophaga*, in which threads of chitin, with an annular space around them, are found within the pore canals and can be drawn out from below. Dennell (1947*b*) has confirmed the existence of such fibres in Crustacea. In the hard exocuticle of *Tenebrio* the canals appear to contain solid sclerotized material free from chitin (Wigglesworth, 1948); while in the outer part of the endocuticle of the *Diataraxia* larva (Lepidoptera) they contain filaments of sclerotized material including chitin with a considerable annular space around (Way, 1948; cf. Kuwana, 1933).

But there is equally no doubt that during the deposition of the cuticle, which is the time when they play their most active part in the physiology of the integument, the pore canals contain cytoplasmic filaments continuous with the epidermal cells. That is so in Crustacea (Verne, 1921), in the young larva of *Sarcophaga* (Dennell, 1946), in *Rhodnius* (Wigglesworth, 1933, 1947*a*) and in *Tenebrio* (Wigglesworth, 1948). It may be that in many insects this state persists. In Diplopoda the contents of the pore canals give no chitin reaction and disappear under the action of pepsin (Langner, 1937). In *Periplaneta*, Richards & Anderson (1942) obtained no evidence of cuticular material in the lumen of the pore canals. In the *Rhodnius* nymph the pore canals remain active in the transport of wax to the surface long after the cuticle is fully formed, which they could hardly do if they were filled with cuticular substance (Wigglesworth, 1945). When the fresh cuticle is dried the pore canals often come to be filled with air (Braun, 1875; Tullberg, 1881; Hass, 1916, in Crustacea; Wigglesworth, 1933, in *Rhodnius*; Richards & Anderson, 1942, in *Periplaneta*); but as Tullberg (1881) and Dennell (1947*b*) point out, this could be due to differential contraction of the substance of the cuticle and the contents of the canals.

7. '*Sekretschicht*'

As will be shown later, the 'cement layer' over the epicuticle is the product of dermal glands and is discharged after the cuticle is complete (p. 435). It has likewise been claimed that most of the Carabidae and Cicindelidae (with the exception of the Mantichorini group) differ from other beetles in having a thick 'Sekretschicht' or 'Sekretrelief' on the surface of the cuticle, that this layer is responsible for much of the pigmentation and that it is poured out from dermal glands. This layer, it is said, may make up as much as one-third of the total thickness of the elytra (Schultze, 1913). It may be wholly responsible for the pigmentation, or it may be superimposed upon the usual exocuticle or 'Pigmentschicht' (Sprung, 1932; Stegemann, 1929, 1930). The 'Sekretschicht' is said to be structureless and to be slowly dissolved in 8% caustic potash, whereas the 'Pigmentschicht' shows the hexagonal structure imprinted by the epidermal cells and resists solution in dilute potash.

Kremer (1920) and Kühnelt (1928*c*), on the other hand, maintain that there is no histological or clear experimental distinction between the 'Sekretschicht' and the 'Pigmentschicht'. They point out that the glands producing this layer have not been demonstrated nor the secretory process described. In the light of recent knowledge about the 'cement layer' of the cuticle the matter requires reinvestigation.

8. *Fine structure of the cuticle*

It was believed by Bütschli (1898) that the endocuticle of insects, including the filaments in the pore canals, had a foam-like structure, an opinion supported by his student Sukatschoff (1899). But to-day it is generally accepted that the cuticle is essentially fibrillar. This is particularly evident in the criss-crossing 'Balken' of beetles. These are strongly birefringent, and are brought out very clearly in polarized light (Biedermann, 1903)—they show positive form-birefringence in the direction of their long axis (Gonell, 1926). If an X-ray photograph is taken of the whole beetle cuticle, it shows only a ring diagram; but if a single 'Balkenlage' is isolated (by treatment with glycerol containing 25% of hydrochloric acid (Künike, 1924)), a beautiful fibre diagram is obtained. The 'Balken', in fact, consist of crystallites (micellae) orientated in the long axis of the strands (Gonell, 1926).

In the endocuticle of other insects the micellae tend to lie at random with the long axis in the plane of the cuticle, so that this shows no birefringence when seen in surface view, whereas cross-sections of the cuticle are strongly birefringent (Schmidt, 1934; Langner, 1937). It is possible (by analogy with plant cell walls (Preston & Astbury, 1937)) that the micellae in a given lamina are all orientated with their long axes parallel, as in the 'Balkenlagen' of Coleoptera; but this is not necessarily the case, as is shown by Picken, Pryor & Swann (1947) on the cocoon of *Donacia*, in which two preferred orientations exist in the thinnest lamina that has been isolated. When the cuticle is examined in section with the electron microscope a very fine lamination becomes visible, light and dark layers alternating. In the cockroach there are from three to five dark laminae per micron. Clearly there are differing molecular densities among the micellae in successive layers (Richards & Anderson, 1942).

Similarly, in the bristles arising from the cuticle, the micellae are orientated in the long axis. In *Drosophila* the bristles show fibrillar ridges which are visible microscopically. From a study of the birefringence of such bristles it is evident that the material of the ridges is more highly orientated than the intervening substance (Lees & Picken, 1945). We shall be considering the micellar structure of the cuticle in greater detail when we deal with its chemistry (p. 418).

9. *General properties of the cuticle*

The integument of insects belongs to the electropositive group of substances and often becomes electrically charged as a result of friction against the environment or of one part against another. But these charges do not appear to have any biological significance (Heuschmann, 1929). The important properties of the cuticle are rather (i) its flexibility and elasticity, which allow of movement, distension and growth; (ii) its hardness, which is developed in appropriate places to provide a rigid support for the attachment of the muscles, to form such horny appendages as the mandibles and claws, or to build such protective coverings as the head capsule, the prothorax, or the elytra of beetles; and (iii) its impermeability, particularly its impermeability to water, which has enabled insects, in spite of their small size and relatively enormous surface area, to colonize very dry environments.

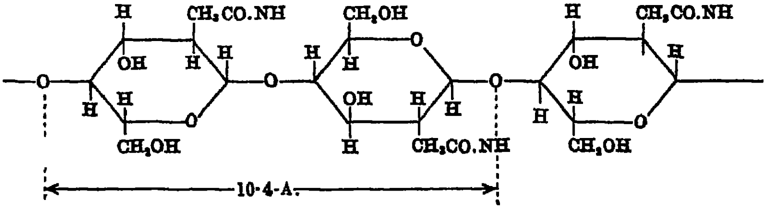
Perhaps the most useful way in which to review the insect cuticle will be to consider the physico-chemical bases on which these properties rest, the mechanisms by which the structural components are laid down, and lastly the physiological activities of which the completed integument is capable—for in the last analysis the integument is still part of a living organism.

II. ELASTICITY AND FLEXIBILITY OF THE CUTICLE: CHITIN AND PROTEIN

1. *Chitin*

The characteristic constituent of the arthropod cuticle, usually held responsible in the main for its tough elastic properties, is chitin. This was the name given by Odier (1823) to the material in the elytra of the cockchafer which resists solution in caustic potash. Bütschli (1874) emphasized the resemblance of chitin to cellulose, and that similarity has become still clearer in recent years (Meyer & Wehrle, 1937).

Regarded by Offer (1907) as polymerized acetylglucosamine (it yields acetic acid and glucosamine in equimolecular quantities on acid hydrolysis), its accepted structural formula as a chain of acetyl glucosamine residues was proposed by Meyer & Mark (1928), Bergmann, Zervas & Silberkweit (1931) producing further evidence that it consists of *n*-acetyl glucosamine residues in which the lactol group of each residue is attached to the 4-position of the next. Chitin is, in fact, identical with cellulose except that the secondary —OH at C_2 is substituted by an acetamide group, thus:



Chitin from the cell walls of fungi, from the cuticle of arthropods, and elsewhere in the animal kingdom, is usually regarded as the same substance with a specific gravity of 1·398 and a refractive index for red light between 1·550 and 1·557 (Sollas, 1907). None the less, the complete identity of chitin from different sources is not universally accepted. Some specimens are more readily dispersed than others (Richards, 1947). The chitin of *Limulus* was said by Fraenkel & Jellinek (1927) to contain more carbon and less nitrogen than that of Crustacea, but this claim was not confirmed by Lafon (1943).

2. *Chitosan*

It was observed by Hoppe-Seyler that chitin heated in saturated caustic potash at 180° C. gives rise to a substance, named chitosan by Ch. Fischer, which retains the form of the original chitin but differs in being soluble in 3% acetic acid and reprecipitated unchanged by alkali. Chitosan is chitin less acetic acid (Araki, 1895); the acetyl groups are probably detached from the surface of the chitin micellae, while many of those in the interior remain (Meyer & Mark, 1928). Chitin in the endocuticle may give a violet colour with zinc chloride and iodine (Zander, 1897) (probably because it has been partially converted into chitosan), but chitosan invariably gives an intense violet with iodine in acid solution, and the production of chitosan constitutes the most reliable test for chitin.

This test was proposed by van Wisselingh for the demonstration of chitin in the cell wall of fungi and was later applied by Wester (1910) to the cuticle of arthropods. The material is heated in saturated potassium hydroxide in a glycerol bath at 160° C. until colourless. It is washed in 90% alcohol, dilute alcohol and water. On treatment with 0·5% iodine in 1% sulphuric acid it then gives a deep violet colour (Campbell, 1929). A confirmatory test was proposed by Brunswik (1921): chitosan freed from alkali is heated carefully to boiling in 10% sulphuric acid and allowed to cool very slowly. Sphaerocrystals of chitosan sulphate are obtained; these still colour violet in iodine and show a white cross in polarized light between crossed nicols. According to Meyer & Mark (1928) chitosan itself is a highly polymerized substance; but it is probably extensively hydrolyzed by this treatment, giving rise to lower saccharides which form crystalline salts with the acid. The true nature of chitosan is not known. Löwy (1910) concluded that it is a polymerized monoacetyldiglucosamine, half the acetyl groups having been removed from the chitin; but Clark & Smith (1936) find that it has insufficient nitrogen for this formula, and suggest that the amine group may have been replaced by a hydroxyl.

Another test for chitin was proposed by P. Schultze (1922, 1923, 1924, 1926). It consists in the treatment of the material with 'Diaphänol' (a solution of chlorine dioxide in 50% acetic acid) in the dark for several days. Chitin, even the most encrusted chitin in the black integument of beetles, will become soft and colourless and will give a violet colour with zinc chloride and iodine. As a test for chitin, however, this method is not entirely reliable. For example, the cuticle of the silkworm and other caterpillars, which are known to contain chitin, sometimes give negative results (Schultze, 1924; Campbell, 1929; Künike, 1924; Kühnelt, 1928*a*).

3. *Protein*

It was recognized by Odier (1823) that chitin makes up only a relatively small part of the insect cuticle. The elytra of *Melolontha* contained about 29%. There was a certain amount of ash and some oils (probably derived from the cellular remnants inside the elytra), but the bulk of the non-chitinous substance was considered by Odier to be protein. Wherever chitinous structures occur, protein is also present (Krawkow, 1893).

Recent work substantiates this view—indeed, Forbes (1930) and Richards (1947) go so far as to regard protein rather than chitin as the fundamental constituent of the arthropod cuticle. Chitin forms about 35% of the dry weight in the abdominal sclerites of *Periplaneta* (Campbell, 1929); 20% of the wet weight or 42% of the dry weight of the cuticle of *Sarcophaga* larvae (Dennell, 1946) (52% according to Fraenkel & Rudall, 1947). Tauber (1934) obtained values of 37·6% in the dorsal abdominal cuticle in *Periplaneta*, 18·2% in the hind wings. Using the diaphänol method, Koch (1932) found 48% chitin in the elytra of *Melolontha*, 64% in the cuticle of *Pieris* and 38% in the abdominal sclerites of *Periplaneta*. In a large number of insects, in which the cuticle was treated with 10% potassium hydroxide at 100° C. until the residue was of constant weight, Lafon (1943) found an average value of 33% chitin, ranging from 55% in the larva of *Calliphora* to 25% in the hemielytra of *Cercopis*. Pepper & Hastings (1943) report the surprisingly low figure of 1·4–2·3% of the dry weight for the chitin in the exoskeleton of the sugar-beet webworm, *Loxostege*.

The bulk of the non-chitinous material in the cuticle is protein. In the insects studied by Lafon (1943) the protein content varied from 25 to 37%. For example, in the puparium of *Lucilia* the total nitrogen is about 10%, of which 20% is chitin nitrogen and 50% protein nitrogen (Lafon, 1941). (The nitrogen content of protein is usually about 16%, and of chitin 6·5%.) In the other insects studied the total nitrogen varied from 9·66% in the elytra of *Lucanus* to 13·3% in the hemielytra of *Pyrrhocoris*. Evans (1938) obtained values for protein soluble in 1% sulphuric acid as high as 60% of the dry weight in the cuticle of *Tenebrio*. In decapod Crustacea, Drach & Lafon (1942) found 40–45% of chitin and 35% of protein in the pre-exuvial cuticle, 75% of chitin and 11% of protein in the post-exuvial.

The extractable protein of the cuticle of arthropods, named 'arthropodin' by Fraenkel & Rudall (1947), has certain constant and peculiar characters. It is highly soluble in hot water, and after precipitation in 10% trichloroacetic acid it redissolves on heating (Fraenkel & Rudall, 1940). More detailed study by Trim (1941) has shown that in the larval cuticle of *Sarcophaga* and in the larva of *Sphinx ligustri* there are two proteins present: (i) The water-soluble 'arthropodin' forms much the greater part; it contains no carbohydrates; in general properties it is akin to sericin (silk gelatin), though the serine and glycine contents are low; tyrosine and tryptophane are present. (ii) The other protein, present in small amounts only, is extractable with 5% sodium hydroxide; it contains a considerable amount of carbohydrate. The cuticular proteins of insects contain no sulphur, being in marked contrast, in this respect, with the proteins of the shell of some insect eggs and of the cuticle of *Limulus* which contain a few per cent of sulphur (Lafon, 1943).

Trim draws an analogy between the protein-chitin association in the cuticle and the sericin-fibroin complex of raw silk. He suggests further that there may be some chemical similarity between the protein-chitin complex and the muco-polysaccharide-protein complexes which occur in the skeletal tissues of other animals. Indeed, recent reviews of polysaccharide chemistry (Stacey, 1943; Haworth, 1946) suggest that the arthropod cuticle may well be regarded as a mucoprotein. The polysaccharide fraction (the polyacetylglucosamine or chitin) may vary in amount in different species or in different regions of the integument. Such a view would be consistent with the findings of Richards (1947) on the apparent absence of chitin from some butterfly scales, and with those of Picken (1948) showing the progressive incorporation of chitin into the scales of *Ephestia* as they mature.

4. *Micellar structure of the cuticle*

We are now in a position to consider in greater detail the fine structure of the cuticle. It is generally accepted that chitin is made up of micellae consisting of bundles of long-chain polymers held together by secondary valences. The micellae have in fact a crystalline structure in which the molecules are geometrically arranged. The dimensions of the rhombic cell, containing four chains, which forms the unit in this system, as it occurs in a chitinous tendon of *Palinurus*, are given by Meyer & Pankow (1935) as $a = 9{\cdot}40$ A., $b = 10{\cdot}46$ A., $c = 19{\cdot}25$ A., where b is the recurring interval in the long axis of the chitin chains (see formula, p. 416)—which coincides with the long axis of the tendon (cf. *Homarus*, Clark & Smith, 1936). These values are in general agreement with those obtained by Gonell (1926) in an isolated 'Balkenlage' of *Melolontha*, where the chains are likewise orientated in the long axis of the strands.

As we have already seen (p. 415) these micellae are orientated also in the long axis of chitinous hairs (Lees & Picken, 1945). For example, in *Astacus* (Castle, 1936), the chitinous hairs show positive birefringence with respect to the axis of the hair; the greatest index of refraction is parallel to the long axis; and this is largely caused by the arrangement of the elongated chitin micelles parallel to this axis ('form birefringence').

In the endocuticle of the *Calliphora* larva, under X-ray analysis, the crystallites show an entirely random orientation in planes parallel to the surface (although as seen under the polarizing microscope the endocuticle is slightly birefringent and the greater refractive index is transverse to the long axis of the body (Picken, 1948)). They can, however, be orientated in any desired direction by compression or extension of the cuticle. Thus when the larva rounds up before forming the puparium there is a 12% increase in its circumference, and the consequent stretching improves the transverse orientation of the crystallites; and this in turn is reflected in the tendency for the fully formed puparium to split in this direction (Fraenkel & Rudall, 1940).

From a further study of the X-ray diffraction and swelling properties of the cuticle of the larva of *Sarcophaga*, Fraenkel & Rudall (1947) suggest that within the micellae chitin is intimately associated with protein. They point out that the cuticular protein, arthropodin, extracted in cold water at 0° C., exists in the fully extended β-configuration. In this form the protein chains may be expected to fit much better with the chitin chains; for three amino-acid residues will give a periodicity of $3{\cdot}4 \times 3 = 10{\cdot}3$ A., which agrees well with the length of the chitobiose unit (10·4 A.). These authors suggest that a part of the X-ray diffraction picture obtained with fresh cuticle may be owing to the proteins present, and that in view of the close dimensional agreement between the protein and chitin chains, the peculiar configuration of intact cuticles may be interpreted in terms of an intimate association of protein and chitin. The chitin: protein ratio in insect cuticle is commonly around 55:45. They suggest that the cuticle may consist of alternating monolayers of protein and chitin; it may be that the monolayer of β-protein synthesizes the new chitin layer—the two structures thus forming interpenetrating lattices.

5. *Metallic colours in the cuticle*

The iridescent colours which occur in many insects are almost all due to interference in the reflexion of light from multiple thin plates. As was shown by Süffert

(1924), in the scales of some butterflies (*Urania* type), there is a horizontal lamination of the scale surface; in others (*Morpho* type) there is an inclined lamination of the high glassy ridges that run along the scale. These conclusions have been confirmed, and the detailed form of the overlapping lamellae in *Morpho* scales have been further elucidated with the electron microscope (Anderson & Richards, 1942; Gentil, 1941, 1942; Kinder & Süffert, 1943).

Thin plates are responsible for the colours on the green scales of *Thecla rubi* and certain papilionids (Schmidt, 1942). In the cassid beetle *Aspidomorpha* the laminae which give rise to the metallic colours occur in a fine cuticular membrane forming the innermost layer, the last to be laid down, in which the usual 'Balken' are wanting. These colours disappear on drying, as the result of closure of the interlaminar spaces which normally contain water, and they are destroyed by pressure (Schmidt, 1941). In this respect they resemble the colours of metallic pupae in Lepidoptera and differ from the butterfly scales, in which a film of air exists between the laminae. Likewise in *Lucilia* and *Phormia*, desiccation at 100° C. causes the colour to change from metallic green to dark blue, suggesting an effect on the separation of thin plates (Lafon, 1943), though here it may well be that a single film on the surface is responsible for the colour (Pryor, 1946). And in Chrysididae and other Hymenoptera there is good evidence that the metallic colours are produced by superimposed platelets (Frey, 1936).

The laminae responsible are not those visible histologically, but are submicroscopic layers (Schmidt, 1941). It seems unlikely that such an exact periodic structure should be the result of cyclical secretion by living cells. It is much more probable that it arises spontaneously by crystallization within the substance of the cuticle; and this is borne out by the fact that periodic structures, producing iridescent colours, exist commonly in cuticular membranes in non-metallic insects. For example, the puparium of blowflies viewed from within shows such interference colours (Pryor, 1940*c*). And along the margins of the scales of *Ephestia* and *Ptychopoda* there are visible with the electron microscope periodic projections apparently homologous to those which produce the colours of *Morpho*. In fact the highly differentiated structures in the iridescent scales appear to be a specialization of structures already present in small normal scales (Kühn & An, 1946). Observations on the serration of the ridges on the bristle-like processes of *Thais polyxena*, and the fibrils running off obliquely from the ridges on the spatulate distal ends of the scent scales of *Trepsichrois mulciber*, also suggest that a lamination of the ridges at an angle inclined to their long axis is common to all scales (Picken, 1948).

It is generally assumed that the iridescent scales, like the ordinary scales, are chitinous structures. But Richards (1947) has recently shown that some of them, such as the highly iridescent scales of *Morpho*, are completely dispersed in strong alkali and give no chitosan reaction. This does not necessarily mean that chitin is wholly absent from these scales; it may only mean that the chitin component is not sufficiently voluminous to form a coherent skeleton.

6. *Conclusion*

The physical properties of chitin are thus very similar to those of cellulose. Where the chain molecules are associated in elongate microcrystalline aggregates, each made up of a number of parallel chains, as in tendons, 'Balken' or bristles, it shows great tensile strength in the direction parallel to the long axis of the chains,[*] combined with low extensibility. Where the aggregates, associated with a soft protein, are irregularly arranged in parallel superimposed lamellae, a tough elastic fabric is produced in which the long chains are orientated by tension, but slowly redistribute themselves in a random manner when the tension is removed.

III. RIGIDITY AND PIGMENTATION OF THE CUTICLE: SCLEROTIN, MELANIN AND LIME

1. *Composition and physical characters of the hard cuticle*

It was recognized by Odier (1823) that chitin forms a comparatively small part of the horny cuticle of insects, and this conclusion has been borne out by recent work. In the scarabaeid larva *Oryctes* the hard cuticle of the head capsule has substantially the same chitin content (32·3 %) as the soft cuticle of the abdomen (36·6 %) (Lafon, 1943). But often the hard cuticle contains much less chitin than the soft. The endocuticle of the cockroach contains 60 % chitin, the exocuticle 22 % (Campbell, 1929). The cuticle of the feeding larva of *Tenebrio* contains 28 % of chitin, the exuviae of the same larva, in which almost all the endocuticle has been reabsorbed, contain only 14·6 % (Lafon, 1947). The dried larval cuticle of *Sarcophaga* is said by Fraenkel & Rudall (1947) to contain 60 % of chitin; this falls to 47 % in the puparium. The corresponding figures for *Calliphora*, given by Lafon (1943), are 54·8 % in the larva and 32 % in the puparium. It was on these grounds that Ferris & Chamberlain (1928) proposed that hard cuticles should be described as 'sclerotized' rather than 'chitinized'.

The formation of the hard cuticle has been widely attributed to impregnation of the chitin framework with insoluble materials, 'Inkrusten', amber or brown in colour. These were believed by some authors to be carbohydrates (Schultze, 1922, 1923, 1924, 1926; Kühnelt, 1928*c*). Later the material was thought to be identical with the 'cuticulin' (p. 427) which forms the basic layer of the epicuticle and which was believed to permeate the chitin and protein of the exocuticle (Wigglesworth, 1933). It is now generally accepted that the substance concerned is protein. There is a large increase in the protein of the cuticle in fly larvae before the hardening of the puparium begins. In *Sarcophaga* the cuticle increases in weight by 6·3 % at this time (Fraenkel & Rudall, 1947). In *Lucilia* the amino-nitrogen in the blood falls rapidly before hardening, as the nitrogen in the cuticle increases (Evans, 1932). In *Calliphora* at this same time the total nitrogen in the cuticle rises from 9·78 to 11 % as the percentage of chitin falls (Lafon, 1943).

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[*] Schulze (1926) gives the tensile strength of chitin as 58 kg./sq.mm., as compared with 14·5 for wool, 35·6 for silk and 50 for drawn copper.

During the hardening of the puparium of *Calliphora*, the mobility of the crystallites is lost; they become firmly anchored in their transverse orientation (Fraenkel & Rudall, 1940). We have seen that by virtue of the arrangement of the chitin micellae, the endocuticle shows 'form birefringence'. The exocuticle is normally isotropic; but it becomes as birefringent as the endocuticle if the amber material is removed by boiling in potash. Clearly the intermicellar spaces of the exocuticle are completely filled with material of refractive index about 1·54, the refractive index of chitin. The chitin is bound by this amber substance like cellulose with lignin (Pryor, 1940*b*).

The hardening of the blowfly puparium was attributed by Fraenkel & Rudall (1940) (i) to a close packing of the orientated micelles, associated with a loss of water from the cuticle (the water content of the cuticle falls from about 70% in the larva to 40% in the puparium; later it may fall to 12% (Fraenkel & Rudall, 1940)), and (ii) to some change in the protein rendering it insoluble; the protein in the larval cuticle, amounting to some 33% of the dry weight, becomes completely insoluble in the puparium.

2. *Sclerotin*

It has long been recognized that this change in the impregnating material results from the activity of an oxidizing enzyme. The cuticle of the newly emerged *Cicada* will not darken in boiled water (Gortner, 1911), nor does the puparium of *Calliphora* become hard if oxygen is excluded (Dewitz, 1916). The true nature of the change was demonstrated by Pryor (1940*a*, *b*).

Pryor first studied the oötheca of the cockroach, which resembles the exocuticle in outward appearance but contains no chitin. It consists of protein in which there is no orientation of the polypeptide chains (as indicated by polarized light and by X-ray diffraction), but the chains are held together by primary valence cross-linkages so that the protein becomes highly insoluble and resistant to swelling. A watery solution of protein is secreted by the left colleterial gland. The right gland secretes a solution of protocatechuic acid (Pryor, Russell & Todd, 1946). On mixing these secretions the protein becomes hard and dark. In the presence of oxygen, which is necessary for the process, and polyphenol oxidase, which is present in the secretion, the protocatechuic acid is oxidized to the corresponding quinone which tans the protein, linking adjacent polypeptide chains, blocking the reactive amino or imino groups and converting a soft and soluble protein into a hard, tough, dark, insoluble material. For this naturally tanned protein Pryor proposed the name 'sclerotin'.

The same theory was then extended by Pryor (1940*b*) to the hardening of the cuticle. The existence of a reducing substance in the insect cuticle was described by Mirande (1904); but this was thought to be glucose and its true significance was not realized. Bhagvat & Richter (1938) discovered that polyphenol oxidase occurs plentifully in the cuticle of many arthropods, and they suggested that the ortho-quinones, produced by this enzyme acting upon dihydroxyphenols, might be of some physiological importance. The part they play was made clear by Pryor.

Using the argentaffin reaction, the reduction of ammoniacal silver hydroxide (Lison, 1936), as a test, Pryor showed that in the pupa of *Ephestia* at the time of browning and hardening, dihydroxyphenols are added to the chitinous cuticle; and similarly in the *Calliphora* puparium, the argentaffin reaction becomes strongly positive during the stage of impregnation (Pryor, 1940*b*).

The precise nature of the phenolic substances responsible is not known (Kuwana, 1940). Dihydroxyphenylacetic acid was isolated from the cuticle of *Tenebrio* by Schmallfuss & Bussmann (1935). Pryor *et al.* (1947) have obtained protocatechuic acid from *Calliphora* puparia, dihydroxyphenylacetic acid and dihydroxyphenyllactic acid respectively from two batches of *Tenebrio* adults, but they failed to detect any phenolic substance in puparia of *Lucilia*. They point out that these are the stable products to be expected if the polyphenol oxidase acts upon tyrosine to give dihydroxyphenylalanine ('dopa') which is then degraded. These phenols are presumably oxidized to quinones which unite with the protein chains to form stable cross-linked structures in which the nitrogen originally present in the free amino groups becomes directly attached to the aromatic nuclei. The presence of sulphur in some cuticular proteins (as in the egg shell or in *Limulus* (p. 418)) does not exclude this method of hardening, since the quinones will react as readily with sulphydryl as with amino groups. Naturally there is much contraction during the process; the outer endocuticle of *Sarcophaga*, for example, being reduced from 150μ in thickness to 70μ (Dennell, 1947*a*).

3. *Enzymic changes during hardening*

The details of the hardening process as it occurs in the puparia of *Calliphora* and *Sarcophaga* have been further studied by Dennell. The amino-acids tyrosine and tryptophane accumulate in the outer endocuticle (the part which becomes sclerotized) in the pupating larva of both species; and Dennell (1946) suggests that they may be directly involved in the hardening. The increase in weight of the cuticle at this time (which reaches a maximum value of 8·8%) agrees with the fall of tyrosine in the blood (which shows a maximum equal to 9·1% of the cuticle weight) (Fraenkel & Rudall, 1947). During hardening, the total tyrosine in the whole larva of *Sarcophaga* decreases from 1·70 to 0·87%—which supports the view that the natural phenol responsible for hardening and darkening results from the oxidation of blood tyrosine (Trim, 1941).

Meanwhile, in the mature larva, the argentaffin reaction becomes intense in the inner epicuticle of *Calliphora* and the outer epicuticle of *Sarcophaga* (Dennell, 1946). In the young puparium of *Sarcophaga* the ortho-dihydroxyphenol (demonstrated by means of the green coloration it gives with ferric chloride, turning red in sodium carbonate) accumulates only in the outer endocuticle, being passed in just when hardening begins. Hardening commences in the inner epicuticle and spreads inwards through the outer endocuticle, ending abruptly where this joins the inner endocuticle. Finally, the inner epicuticle becomes indistinguishable in sections from the hardened outer endocuticle or, as it is now called, the exocuticle.

The oxidizing enzyme, the polyphenol oxidase, appears to be elaborated in the epidermal cells and later concentrated in the inner epicuticle—as judged by the intense blue coloration it gives with Ehrlich's 'Nadi' reagent. That explains why hardening and darkening proceed inwards although the phenol is supplied from within. The polyphenol is presumably oxidized to orthoquinone at the inner epicuticle. The quinone then appears to diffuse into the cuticle or, perhaps, itself oxidizes the advancing polyphenol so that a wave of quinone formation spreads inwards (Dennell, 1947*a*).

The complete absence of quinone and phenol from the inner endocuticle is very striking. It results from the fact that, whereas the inner epicuticle is a region of high oxidation-reduction potential, the inner endocuticle is actively reducing. Hence the formation of orthoquinone in the inner endocuticle is inhibited. (Hurst (1945*b*) has put forward the view (for which the evidence has not yet been published) that local differences in hardening of the cuticle result from differences in the entry of catalase which destroys hydrogen peroxide and so diminishes the activity of the peroxidase.)

4. *Tyrosine and the darkening of the cuticle*

Protein tanned by quinones is always dark in colour. This coloration may be due to the presence of chromophore groups, such as the quinonoid group, in the molecule, or it may be due to coloured by-products arising from the oxidation of phenols not attached to any protein chain. Whatever the cause, the presence of 'sclerotin' is always associated with a dark colour; hard cuticles are always brown or black[*] (Pryor, 1940*c*). The question arises whether there is a formation of true melanin alongside the sclerotization.

The tyrosine content of the larva of *Sarcophaga* just about doubles during the 48 hr. before pupation, and then falls steeply. Fraenkel & Rudall (1947) consider that this tyrosine is very largely deaminated and utilized in hardening, but that some undeaminated tyrosine probably serves for melanin formation. The same view is taken by Trim (1941), who shows that the tyrosine in the cuticle of *Sarcophaga* falls from 3·5 to 2·0% on pupation, that in the whole organism decreasing from 1·70 to 0·87%. In *Drosophila*, also, there is some evidence that in addition to the darkening associated with the tanning of the cuticle, there is a blackening due to the deposition of melanin, and that the two processes can be differentially influenced by different genes (Waddington, 1941).

In the full-grown larva of *Sarcophaga*, tyrosine and tyrosinase are present together in the blood, and this quickly blackens if it is shed. But blackening of the blood does not take place *in vivo*. According to Dennell (1947*a*) that is because the oxidation of tyrosine is inhibited by the low oxidation-reduction potential in the blood, and this in turn is ascribed to the action of a dehydrogenase acting upon some unspecified hydrogen donator. There is a steady fall in the oxidation-reduction potential as tyrosine and tyrosinase appear in the blood; an abrupt rise coincides with the initiation of puparium formation. Tyrosinase activity is thus released, dihydroxyphenol (probably 'dopa') is produced, and hardening and darkening can proceed. The tyrosinase is apparently secreted by the oenocytoids which appear in the blood late in the last larval stage and disappear before pupation.

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[*] There is one striking exception: the elytra of *Stenocara*, which are very hard but white. In most beetles the outer wall of the elytron is hard and dark, the inner thin and colourless. In *Stenocara* the thick outer wall is colourless and is responsible for the whiteness; but the inner wall is now thickened and black, and it provides the rigidity. It is an exception which proves the rule (Pryor, 1948).

5. *Lime*

In the cuticle of Crustacea, hardening is achieved mainly by the incorporation of lime, but hardening of the same type as in insects occurs in the epicuticle (Drach, 1939); and Pryor (1940*b*) and Dennell (1947*b*) produce evidence that here, and in the claws and bristles, there is a tanning reaction as in insects.

Among the insects themselves, lime is used comparatively seldom in the hardening of the cuticle. The ash content is only around 2–5% (Beauregard, 1885; Lafon, 1943). It is best known in the cuticle of stratiomyid larvae (Leydig, 1860; Viallanes, 1882). In the larva of *Sargus*, for example, lime occurs in the form of rhombic plates with rounded angles embedded like warts ('Kalkwartzen') in organic material on the surface of the cuticle, with groups of pore canals running up to them. The lime in these larvae makes up 75·9% of the weight of the skin (Müller, 1925; Krüper, 1930). (In the cuticle of *Astacus*, lime is said to make up 48·5%.)

In the puparium of the cherry fly, *Rhagoletis cerasi*, there is an epicuticle 0·75 μ thick, an exocuticle 1 μ thick and an endocuticle of 25–29 μ, made up of three layers. The outer of these (15–25 μ) is filled with refractile granules consisting of calcium carbonate with a trace of silicate, which make up more than half the weight of the puparium (Wiesmann, 1938). In the celery fly, *Acidia heraclei*, the lime is simply poured out from the Malpighian tubes below the surface of the puparium where it forms an inner shell (Keilin, 1921). In these Trypetidae the puparium, although hard, is quite white. Impregnation with lime has taken the place of phenolic tanning, the argentaffin reaction is absent, and if the lime is removed with acid, the cuticle becomes limp and transparent (Pryor, 1940*c*). It is noteworthy that cave-dwelling Crustacea are likewise white, whereas the cave-dwelling insects, with their tanned cuticle, are necessarily of the same brown tint as normal forms (Pryor, 1948).

6. *Conclusion*

We may end this section with some reflexions borrowed from Pryor (1940*c*). For a terrestrial animal, whose surface is subject to desiccation, it is desirable to have a 'dry' skeletal protein of the type of keratin or sclerotin whose rigidity is dependent upon a large number of primary valence cross-linkages. The rigidity of such proteins is, to a great extent, independent of the presence of water; they can therefore lose water without suffering any deformation in shape. As in the integument of vertebrates keratin and collagen are in some degree complementary, so in insects sclerotin and chitin play complementary roles. The way in which the three-dimensional molecular network formed by sclerotin is combined with the fibrillar structure of chitin, suggests a comparison between the composition of the insect skeleton and that of certain artificial plastics, in which the cellulose fibres are impregnated with a phenol-formaldehyde resin. The synthetic resin is used in an unorientated form to stabilize the cellulose fibres so that they can carry compressive as well as tensile load, and to bond the fibres together so that load may be transmitted from one fibre to another (Smith, 1943). That is precisely the function of the sclerotin in the exoskeleton of insects. It was the evolution of this remarkable chitin-sclerotin fabric which made flight possible for the insect.

IV. PERMEABILITY OF THE CUTICLE: WAX, CUTICULIN AND CEMENT

1. *Permeability of the hard cuticle*

The transpiration of water through membranes of keratin or collodion is greatly diminished if the membrane is allowed to dry (King, 1944). This same effect is seen in the insect. Larvae of the wireworm *Agriotes* taken from the soil have suffered so much mechanical injury to their cuticle that they lose water very rapidly. But if exposed in a dry atmosphere the rate of water loss quickly diminishes (Wigglesworth, 1945). Likewise if the epicuticle and part of the exocuticle of the hard pronotum or elytra of *Lucanus* or other beetles are ground away, they are still hydrophobe and impermeable (Lafon, 1943).

Clearly the tanning process with the consequent 'drying' and condensation of the protein can provide some degree of impermeability to water. Indeed, Koidsumi (1934) suggests that a correlation exists between the hardness and blackness of the insect cuticle and its impermeability to water. This idea is elaborated by Kalmus (1941*b*) who discusses at length the relation between ecology and cuticle colour, and formulates a series of 'laws', claiming among other things that dark colour in insects is associated with resistance to desiccation. It was found, for example, that dark mutants of *Drosophila* survived longer in a dry atmosphere than light mutants (Kalmus, 1941*a*). But there are so many exceptions that these generalizations are of doubtful value. Some of the insects most highly resistant to desiccation, such as the larva of *Tineola* (Mellanby, 1934), are colourless; the mealworm, which can withstand an atmosphere of 0% relative humidity at about 20° C. for 30 weeks (Buxton, 1930), is no darker than the wireworm which dries up in 30 min. (Wigglesworth, 1945); and the same applies to insect eggs. It is nearer the truth to say that impermeability to water is independent of thickness and sclerotization in the cuticle (Mellanby, 1934; Kühnelt, 1939; Eder, 1940).

That cuticular tanning is not a very efficient means of waterproofing is seen in the puparium of *Calliphora* which is quite heavily tanned. The fully hardened puparium 24–48 hr. old is highly waterproof; but if the most superficial layer is abraded by rubbing it lightly with alumina dust it loses this property and quickly dries up (Wigglesworth, 1945). This does not happen in the puparium 3 days old; but that is not because the condensed chitin-protein complex of the puparial shell is now waterproof (as suggested by Hurst, 1941), but because the delicate waterproof cuticle of the true pupa has been formed inside (Wigglesworth, 1945).

In many insects, as we shall have occasion to see later (p. 442), the pore canals retain fluid or cytoplasmic contents in the fully hardened cuticle. In such insects the hardening process will not in itself make the cuticle less permeable. But in the hard exocuticle of the adult *Tenebrio*, for example, although the pore canals do not contain chitin, they do appear to be solid, being filled perhaps with sclerotin (Wigglesworth, 1948). In cuticles of this type hardening will increase the impermeability of the outer layers to water.

2. *Epicuticle: 'cuticulin'*

It was recognized by Kühnelt (1928*a*, *b*) that it is the outermost layer of the cuticle of insects, the 'epicuticle', which is responsible for most of the impermeability to water. Indeed, it is the properties of this layer which have enabled the insects to colonize dry terrestrial environments. The epicuticle was defined by Kühnelt as the layer, of the order of 1 μ in thickness, that is not penetrated by the pore canals, contains no chitin, and resists solution in cold concentrated hydrochloric or sulphuric acids; he described it as a 'lipoid' cuticle and believed it to contain cholesterol esters of fatty acids which were responsible for its impermeability. It gives a positive Liebermann-Burchardt reaction for sterols, and, though insoluble in fat solvents, it breaks down in hot alkali or in warm nitric acid saturated with potassium chlorate to give oily droplets which stain with fat stains. It shows a general resemblance to the cuticle of plants. The outer epicuticle of the *Sarcophaga* larva (p. 411), the layer less than 1 μ thick, stains deeply with black Sudan B (even after treatment with lipid solvents) and gives a positive Liebermann-Burchardt reaction, whereas the inner epicuticle of about 4 μ is negative to both (Dennell, 1946).

The first layer to be deposited when the cuticle of *Rhodnius* is being formed is the layer which will become the 'epicuticle' as visible in histological sections. This layer consists of protein and lipid, intimately associated. At first it is soft and colourless; at the time of moulting it becomes hard and amber-coloured. The name 'cuticulin' was proposed for this material (Wigglesworth, 1933). It was supposed to be a mixture of fats and protein, in which the fats became polymerized in the presence of an oxidizing enzyme to form a varnish; and it was this change which was thought to be responsible for the impermeability of the cuticle which develops just before moulting. It was also thought that 'cuticulin' not only composed the epicuticle but permeated the chitin and protein of the exocuticle as the amber material in that layer.

But the amber material was shown by Pryor (1940*b*) to be tanned protein (sclerotin), and he suggested that lipides were secreted on to the surface of the sclerotin and that by virtue of its lipophil properties, which result from the blocking of the hydrophil groups in the tanning process, the lipides impregnate it and are responsible for its impermeability. A similar interpretation was adopted by Hurst (1941).

A reinvestigation of the cuticle of *Rhodnius* showed that, as judged by the liberation of fatty droplets in nitric acid and potassium chlorate, lipids do not occur in quantity in the exocuticle, but are confined to the epicuticle; and here, to a large extent, they are fixed and not extractable by fat solvents. It was therefore concluded that 'cuticulin' is limited to the epicuticle; that it consists of lipoprotein, which is perhaps denatured and condensed, and is finally tanned along with the other proteins of the outer layers (Wigglesworth, 1947*a*). These results have been confirmed in the adult *Tenebrio*, in which it is found that the cuticulin layer is far thicker in the relatively thick ventral cuticle of the abdomen than it is in the very delicate dorsal cuticle (Wigglesworth, 1948).

Richards & Anderson (1942) concluded that the thin outer layer of the epicuticle in the cockroach consists of 'polymerized lipotanned protein', while the thicker underlying layer is composed of tanned protein without lipoid; and Lafon (1943), who isolated cuticulin from the elytra of Coleoptera by treatment with 15% hydrochloric acid at 120° C., is substantially in agreement with these ideas; he estimates that cuticulin makes up about 0·3% of the total integument. The compound containing 10·7% of nitrogen, described by Aronssohn (1910) as the main constituent in the cast skins of bee larvae, is presumably cuticulin.

3. *Epicuticle: waxes*

It is not, however, the cuticulin layer which is responsible for the impermeability of the cuticle to water. It has long been known that insects are caused to dry up if they are exposed to certain fine mineral dusts (Zacher, 1937; Alexander *et al.* 1944; Parkin, 1944). These dusts are effective only if the insect moves in contact with them (Wigglesworth, 1944). They act by abrading from the surface the true waterproofing layer. The sites at which this abrasion occurs can be demonstrated by immersing the living insect in a solution of ammoniacal silver hydroxide. The outer layers of the cuticle contain accessible dihydroxyphenols which will reduce the silver; but the normal insect when so treated shows no staining because an impervious layer separates the silver solution from the cuticulin. Wherever this protective layer has been abraded, there is a patch of deep brown staining. This may affect all the prominent points and the crests of all the folds in the cuticle, or it may be confined to the soft cuticle in the flexible joints (Wigglesworth, 1945, 1947*b*). In such insects there is no injury to the cuticulin layer that can be detected with the microscope.

It was suggested long ago by Keilin (1913) that the dermal glands of certain tipulid larvae secrete a fatty covering which restricts evaporation. The same suggestion was made by Woods (1929) with regard to the glandular hairs of the chrysomelid larva *Altica*; and the smearing of wax over the wings and body by aleurodid flies was supposed by Weber (1931) to serve the same purpose. Perhaps the closely crowded granules of wax secretion which form the 'bloom' on certain aphids, dragonflies, etc., has a restrictive action on transpiration, as the corresponding layer in plants certainly has.

But the first clear demonstration of this function in insects was given by Ramsay (1935). Ramsay found that evaporation from the body surface of the cockroach shows a sudden increase if the temperature rises above 30° C. The impermeability to water is due to an oily or waxy layer on the surface. This material will spread as a film, sufficiently thick to show interference colours, over the surface of droplets of water applied to the cuticle and will prevent such droplets from evaporating. This film remains rigid up to 33° C., it then becomes fluid; and Ramsay suggests that at 30° C. it undergoes a change of phase, either of expansion or melting and allows evaporation to take place.

When observations were extended to other insects it was found in all of them that there is a similar break in the curve relating rate of transpiration with temperature (Wigglesworth, 1945). There is a critical temperature above which the rate of water loss increases abruptly. This temperature may be around 30–37° C. and so be demonstrable in the living insect (for example, in *Blattella*, *Calliphora* larva, the sawfly larva *Nematus*, the cabbage caterpillar *Pieris*), or it may be around 50–60° C. and become evident long after the insect has been killed by the high temperature, as in *Tenebrio* and *Rhodnius*. In general, the critical temperature is low in phytophagous insects from relatively moist environments, high in insects from dry environments. In the larva of *Pieris* the critical temperature is about 37° C.; in the pupa of the same insect, which has to withstand exposure in the open for many months, it is about 58° C.

The materials responsible for this waterproofing of the cuticle can be extracted from the cast skins of the insects with boiling chloroform. They prove to be waxes of the same general type as beeswax, but they vary widely in physical properties. In the cockroach we have to do with a soft grease; in the larvae of *Nematus* and *Pieris* they are soft pale yellow waxes without crystalline form; in the mealworm *Tenebrio* and in *Rhodnius* they are hard, white and crystalline. In the pupal wax of *Pieris* there are yellow and white fractions with different properties (Beament, 1945).

The extracted waxes can be laid down in the form of a thin film upon artificial membranes such as gelatin tanned with benzoquinone, or upon a piece of butterfly's wing from which all lipides have been removed, and they render these materials highly impermeable to water. It appears that under the influence of the substrate membrane the wax molecules of the innermost layer are orientated at right angles to the surface and so closely packed in crystalline form that water molecules will not pass through. If these artificially waterproofed membranes are warmed, they show the same phenomena as the intact insect (Beament, 1945). At a critical temperature there is a sudden increase in the rate of transpiration; and this temperature is approximately the same in the isolated wax on the artificial membrane as it is in the normal insect from which the wax was obtained (Table 1).

There can be little doubt that it is these waxes which are responsible for the waterproofing of the cuticle. By relating the amount extracted with the surface area of the insect, Beament (1945) calculated that they usually form a layer of the order of 0·2–0·3 μ in thickness. The layer in *Nematus* is thinner, about 0·095 μ;

that in the resistant pupa of *Pieris* is thicker, about 0·4μ; and the layer of grease on the cockroach about 0·6μ. The thickness of the wax layer bears little relation to the thickness of the cuticle. The *Calliphora* puparium as shed is 47·0μ thick and carries a wax layer of 0·27μ. The cast skin of the true pupa is 3·1μ thick, with a wax layer of 0·18μ. The waxes have fairly definite melting-points, but the critical temperature for the passage of water lies some 5 or 10° C. below the melting-point. At this lower temperature there is commonly a visible change in the wax, which then becomes less opaque. It seems probable that at the critical temperature the intermolecular spacings in the crystalline waxes increase abruptly and the oscillating molecules begin to rotate freely so that the water molecules can escape (Müller, 1932). At temperatures below their melting-points, films of long-chain compounds may gradually or abruptly assume a more open structure which will allow water molecules to pass through (Ställberg-Stenhagen & Stenhagen, 1945).

Table 1. *Approximate 'critical temperature' for the increase in transpiration through the cuticle of intact insects* (from Wigglesworth, 1945) *and through films of the isolated waxes* (from Beament, 1945)

	Intact insect (° C.)	Isolated wax (° C.)
Blattella	31	30
Calliphora prepupa	35	33
Calliphora pupa	47	48
Nematus larva	34	34
Pieris larva	37	39
Pieris pupa	58	57
Tenebrio larva	49	50
Rhodnius nymph	57	57

(It may be noted here that Schmidt (1939) observed wax in the form of orientated crystals inside the lumen of the cuticular hairs of *Bombus*, and sometimes on the surface of the base of the hairs. This wax can be made visible by its birefringence. The double refraction disappears on warming and reappears on cooling.)

The innermost layer of wax is probably the most effective in restricting the passage of water (cf. Alexander, Kitchener & Briscoe, 1944), and it may well be that the orientated molecules in the surface of the substrate membrane will influence the orientation of the innermost wax molecules. For the different membranes used vary in effectiveness, and the most effective as substrates for a waterproof wax film are those chemically most akin to insect epicuticles: *Pieris* wing > tanned protein > parchment > celluloid (Beament, 1945). The most mobile substances, such as the grease in *Blatta*, will spread progressively over a membrane. They are removed by adsorption when a mineral dust is applied to the surface of the insect (Wigglesworth, 1945), although a thin, perhaps monomolecular, layer remains which cannot be removed in this way. The soft waxes show improved waterproofing after warming up beyond the critical temperature and allowing to cool, perhaps because their molecular orientation is thereby facilitated. They are not removed by adsorption in contact with stationary dusts (Beament, 1945). Both the intact insects and the isolated waxes become more permeable after exposure to chloroform vapour; this is particularly so with the softer waxes.

The chemical properties of the ether extract[*] of silkworm exuviae were studied by Bergmann (1938). It is a wax-like substance which makes up 4–4·5% of the cast skin. It contains from 55 to 60% of non-saponifiable material which gives a very faint and uncertain Liebermann-Burchardt reaction for sterols. The whole is considered to be a mixture of paraffins of the probable order C_{25}–C_{31} and esters of *n*-alcohols and acids, both saturated and unsaturated, of the probable order C_{26}–C_{30}. It is in fact very similar to the waxy substances obtained by Chibnall, Piper, Pollard, Williams & Sahai (1934) from the cuticle of plants. (Rosedale (1946) states that the cast skins of South African locusts have a fat content as high as 70%, with an iodine value of 195.)

The conception of the waterproofing layer as outlined here, where it is considered to be a layer of closely packed wax molecules resting on a foundation of tanned lipoprotein (cuticulin), is not accepted by Hurst (1945*a*). In a preliminary account of work not yet published in full, Hurst suggests a hypothetical mosaic structure for the epicuticle of the larva of *Musca*, consisting of alternating lipoprotein and protein zones, the whole being impregnated with lipides extractable by fat solvents. The loss of water through the epicuticle may be greatly increased if the surface of the insect is smeared with suitable detergents (Wigglesworth, 1945); and Hurst (1941) made the curious observation that *Tenebrio* larvae allowed to remain in contact with *Calliphora* larvae lost water excessively by evaporation. He attributes this to the effect of the more hydrophillic character of the free lipoid on the cuticle of *Calliphora*.

4. *Epicuticle: cement layer*

The waxy materials which form the waterproofing layer are readily soluble in chloroform, but if the intact insect is immersed in chloroform for 5 min. and subsequently treated with ammoniacal silver, there is comparatively little exposure of the underlying layer containing phenols. It was this observation which revealed the fact that in *Rhodnius* the wax layer is covered by a thin protective coating of cement. This layer may sometimes be detached and become visible in sections; in the *Rhodnius* nymph it is readily demonstrated by immersing a fragment of cuticle in concentrated sulphuric acid, when it becomes separated as the cuticle swells (Wigglesworth, 1947*a*). A similar layer covers the wax in the tick *Ornithodorus* (Lees, 1946); and it is very easily seen in the caterpillar of the tomato moth (*Diataraxia*) where the membrane, separated in chloroform, shows the impress of every detail of the cuticle (Way, 1948).

The cement layer has recently been studied more fully in the adult mealworm *Tenebrio* (Wigglesworth, 1948). In the untreated state it will not stain with ammoniacal silver; but if the insect is boiled for 5 min. in chloroform it does show a finely granular silver staining, and after prolonged treatment with boiling chloroform it softens and begins to fuse into rounded silver staining droplets. On these grounds it was concluded that the cement layer probably consists of tanned protein combined with lipides in some form. In the caterpillar of *Diataraxia* the cement layer likewise stains with silver only after treatment with chloroform, and in this insect it stains readily with black Sudan B (Way, 1948). Further details about the cement layer will be given when the deposition of the cuticle is discussed (p. 435).

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[*] Bergmann refers to this material as 'cuticulin', but that is using this term for material quite different from that for which it was proposed.

The function of the cement layer is presumably to protect the wax. In the newly moulted insect, before the cement has been poured out, the wax layer is freely exposed. It is then extremely liable to damage both by mechanical injuries and by wax solvents (Wigglesworth, 1945, 1948). The fully hardened insect with its cement covering laid down is more robust, but where it is subject to severe abrasion, as are insect larvae in the soil, for example, its surface may be so scratched that it is highly pervious to water. Larvae of insects from the soil usually dry up rapidly when removed from their humid environment. The rate of water loss in larvae of *Bibio*, *Tipula*, *Pterostichus*, *Agriotes*, *Aphodius*, *Phyllopertha*, *Agrotis* and *Hepialus* is more or less related with the amount of abrasion which is revealed by immersion in ammoniacal silver (Wigglesworth, 1945). The larva of the wireworm *Agriotes* normally swells or contracts in watery solutions like an osmometer (Evans, 1944), but if it is allowed to moult out of contact with the soil, its cuticle is impermeable and shows a critical temperature for water loss just like other insects (Wigglesworth, 1945).

5. *Entry of water through the cuticle: aquatic insects, etc.*

While most insects show a very great resistance to the loss of water by transpiration, the passage of water from without inwards seems to take place more readily. If the isolated cuticle of *Rhodnius* is reversed, there is a 20-fold increase in the rate of transpiration of water (Beament, 1945). Hurst (1941) records an increase of 100-fold in the larva of the blowfly. No wholly satisfactory explanation of this phenomenon has been published.

In larvae of *Phlebotomus* water is continually taken up from moist surroundings through the skin (Theodor, 1936). Intact grasshoppers (*Acridium*) will absorb water through the cuticle after desiccation (Colosi, 1933), and if drops of water (as well as salt solutions, acetic acid, benzene, etc.) are placed on the leg of the cockroach, droplets of liquid almost instantaneously make their appearance in the trachea below (Rajindar Pal, 1947). But even more striking is the uptake of water vapour from the air. Mealworm larvae (*Tenebrio*) (Mellanby, 1932), grasshoppers (*Chortophaga*) (Ludwig, 1937), flea larvae (*Xenopsylla*) (Edney, 1947) and ticks of many species (Lees, 1946) are able to take up water from the air even when it is well below saturation. This uptake, at least in the case of ticks, has been proved to take place through the cuticle (p. 443).

Comparatively little is known about the permeability of the cuticle in aquatic insects. The cuticle over the general surface of the mosquito larva (*Aedes*) seems to be highly impermeable; larvae show no shrinkage after immersion in hypertonic solutions for 24 hr. But this is true only if the posterior extremity bearing the anal papillae is tied off. The cuticle over the anal papillae is very thin and quite colourless, and water and salts are normally absorbed through them (Wigglesworth, 1932*b*, 1938*b*). On the other hand, the cuticle of chironomid larvae (which differ from the culicids in respiring solely through their body surface) is readily permeable to acids and alkalies, inorganic poisons and diffusible dyes such as neutral red. The degree of permeability varies in different species, independently of the *thickness* of the cuticle; it depends upon the surface layers (Alexandrov, 1935).

The cuticle which lines the foregut and hindgut is likewise permeable to some extent in certain insects. There is a slow diffusion of acids and alkalies through the cuticle of the crop and hindgut in the cockroach, much slower through the crop (5–8μ thick) than through the hindgut (2μ thick) (Eidmann, 1922). The hindgut plays an active part in reabsorbing water from the excreta; the cuticle covering the rectal epithelium must therefore be readily permeable to water (Wigglesworth, 1932*a*); and it has been shown that in *Limnophilus* (Trichoptera) and in *Chironomus*, chlorides also can be taken in by these cells (Boné & Koch, 1942).

6. *Entry of insecticides through the cuticle*

It is not possible to review here the large problem of the entry of insecticides through the cuticle, but a few points which bear upon the physiology of the cuticle may be noted. The cement and wax layers which restrict the passage of water also oppose the entry of insecticides; their entry is enormously accelerated after these layers have been injured by abrasion (Wigglesworth, 1945).

It is generally true that the presence of wax solvents will facilitate the passage of toxic substances into the insect, the epicuticle offering the main barrier (Lennox, 1940; O'Kane, Glover, Blickle & Parker, 1940; Morozov, 1935; Umbach, 1934). Epicuticles most readily stained with Sudan III occur in insects most susceptible to pyrethrum (Klinger, 1936). In the caterpillar *Loxostege* there is a progressive decrease in susceptibility to pyrethrum in oil sprays in successive instars, associated perhaps with the progressive diminution in the fat content of the cuticle (Pepper & Hastings, 1943). The entry of alcohol through the cuticle of *Calliphora* larvae is greatly accelerated in the presence of kerosene, this effect being attributed to the increased permeability of the outer lipid layer in the presence of apolar substances (Hurst, 1940, 1943). Pyrethrum enters the cuticle of *Rhodnius* progressively more rapidly when dissolved in the more volatile paraffins; here the thickness of the endocuticle is important in slowing down the rate of entry. In this insect, oils, particularly in the presence of some oleic acid, will pass through the cuticle and appear in the cells of the general epidermis and in the dermal glands below. They can never be detected in the substance of the cuticle, presumably because they pass through in droplets or particles beyond the resolving power of the microscope (Wigglesworth, 1942*a*).

Where the pore canals, containing fluid or cytoplasmic filaments, come close to the surface of the epicuticle, toxic substances have only a very little way to diffuse before reaching what are in effect the soft tissues of the insect. A paper by Webb & Green (1945) illustrates some of the ways in which the physico-chemical properties of an insecticide and of its carrier solvent will influence this passage through the cuticle. If a material could be incorporated in the solvent, which would disrupt the surface layers and expose the pore canals, it should enhance the effectiveness of an insecticide so far as it enters by this route. Abrasive dusts (alumina, pyrophyllite, etc.) probably have this effect. There is some evidence that detergents which cause most water loss when smeared on the surface of the cuticle are also the most effective in assisting the entry of insecticides. But the existence of the highly resistant cement layer adds to the difficulty of dispersing the underlying wax (Wigglesworth, 1945).

V. DEPOSITION AND MOULTING OF THE CUTICLE

1. *The epidermis and the mechanism of cuticle secretion*

The moulting of the old cuticle and the deposition of the new is preceded by active growth in the epidermis. Dermal glands, oenocytes, trichogen cells, perhaps the cells which form the basement membrane, all arise by differentiation from the epidermis. The epidermal cells become detached and then divide and multiply. Many more cells are produced than are required to form the new cuticle. The unwanted cells degenerate, so that alongside the dividing cells are nuclei in all stages of chromatolysis. Eventually the 'chromatic droplets' derived from these dead nuclei disappear, and the definitive epithelium, with nuclei evenly spaced, is ready to lay down the new cuticle (Wigglesworth, 1933, 1942*b*, 1948).

There has been much controversy in the past as to whether the cuticle is formed by the secretion and discharge of material outside the cell (Haeckel, 1857; Kölliker, 1857; Bütschli, 1894; Tower, 1906), or by the transformation of the cytoplasmic substance of the superficial portions of the cell itself, the product from adjacent cells fusing to form the lamellae (Vitzou, 1882; Chatin, 1892, 1895*a*, *b*; Holmgren, 1902*a*, *b*; Hass, 1916), or by both methods (Vignon, 1901; Plotnikov, 1904; Kapzov, 1911; Korschelt, 1923; Ahrens, 1930). The fact that the surface of the fully formed cuticle often bears the imprint of the cell boundaries (Kölliker, 1857; Viallanes, 1882) and that the little columns of cuticle formed by each cell may still be recognized as cuticular prisms in the fully formed cuticle of some Crustacea, though not in others (Vitzou, 1882; Dennell, 1947*b*), has been used as an argument to support the transformation hypothesis; while the undoubted fact that chitin *can* be discharged as a semifluid secretion (as in the formation of the peritrophic membrane (Vignon, 1901; Wigglesworth, 1930)) has been used to support the secretion hypothesis.

At the present time this controversy may perhaps be considered dead. Up to the time of moulting the pore canals almost certainly contain cytoplasmic filaments extending practically to the surface of the cuticle. The cuticle must be regarded as 'alive' almost to the surface. In many insects, apart from the thin covering of wax and cement, this condition persists. In fact, it seems more reasonable to regard the surface of the cuticulin layer, rather than the junction between the endocuticle and the main body of the cell, as the cell boundary. If that is so, one would have to regard the cuticular material as being laid down within the cell body. But as Biedermann (1903) long ago pointed out there is no fundamental difference between the secretion of a skeletal substance outside the cell (Ausscheidung) and its deposition within (Umwandlung). When the products remain in intimate association with the cell plasma it is impossible to draw any distinction. The chitinization of the contents of the pore canals (Dennell, 1946, 1947*b*) would have to be regarded as transformation within the cell substance. Likewise the formation of the bristles is a clear example of the production of cuticle by the transformation of a part of the body of the cell. The conversion of the muscle insertions into cuticle is another example (Chatin, 1892, 1895*a*, *b*). The production of the laminated cocoon of chitin and sclerotin by the larva of *Donacia* is an example of the extracellular formation of a structure very similar to the cuticle (Picken *et al.* 1947).

✓ 2. *Deposition of the 'epicuticle'*

The epicuticle is defined here as the complex of thin superficial layers which contains no chitin. The deposition of these layers has recently been studied in detail in *Rhodnius* (Wigglesworth, 1947*a*) and in the *Tenebrio* adult (Wigglesworth, 1948). The first to be deposited is the *cuticulin layer* which appears as a thin, apparently homogeneous membrane, over the surface of the epidermal cells. When first formed it gives a positive Millon's reaction, it stains with Sudan B and with osmic acid, and on warming with nitric acid and potassium chlorate it breaks up to liberate oily droplets. It is thought to consist of lipoprotein. The oenocytes, which are small when moulting begins, reach their maximum size and appear to be discharging secretion just before the cuticulin is deposited, and then contract rapidly. At that time the oenocytes stain like the newly deposited cuticulin. It is therefore suggested that they manufacture the lipoprotein which is then taken up by the epidermal cells.

The chitin and protein of what will become the exocuticle is then laid down. It is traversed by the pore canals, and these appear to penetrate the cuticulin layer. For, although it is not possible to see this penetration in ordinary sections, if the fresh new cuticle is immersed in ammoniacal silver hydroxide at this stage, certain of the cells stain an intense brown, and from these cells dark brown filaments run through the cuticle to the surface. Very soon minute silver staining droplets are discharged from the tips of these pore canals, and as they enlarge they run together and fuse until the cuticle is covered with a more or less continuous layer which gives an intense argentaffin reaction. This secretion is semi-fluid; it can be smeared and the droplets caused to run together if the surface is touched with a pointed slip of filter paper. It consists presumably of polyphenols bound to protein, and has been termed the *polyphenol layer*.

Within a few hours before moulting the *wax layer* begins to appear over the surface of the new epicuticle, so that the argentaffin reaction becomes discontinuous, until finally, at the time the old skin is shed, there are only scattered points which still stain with the silver. The wax also is secreted presumably through the pore canals, and then crystallizes on the surface of the polyphenol layer; but nothing is known of the mechanism by which it is held in solution. Beament (1945) found that the hardest cuticular waxes appear to have associated with them highly polar substances which will increase their spreading on water at the transition temperature. He suggests that these substances, perhaps phospholipides, perhaps proteins, serve as solubilizers or emulsifiers.*

At the time the insect sheds the old skin the polyphenol layer is covered, except for a few small spots (Wigglesworth, 1947*a*, 1948). Covering is completed within the next 24 hr. Thus, although the insect is practically waterproof when it moults, there is a small extra loss of water during the 24 hr. or so after moulting (Wigglesworth & Gillett, 1936; Wigglesworth, 1948; Smallman, 1942).

The *cement layer* is discharged from the dermal glands and poured over the surface of the wax within an hour after moulting. At the instant of moulting, the dermal glands are distended with secretion; an hour or so later they are for the most part empty. In the *Rhodnius* nymph, the discharge of the cement is readily recognized by the fact that the cuticle, completely hydrophobe and unwettable in the newly moulted insect, becomes hydrophile within an hour. In *Tenebrio* the same change occurs, but it is not so striking, because the cement layer is itself only moderately hydrophile. The exposed wax of the newly moulted insect is readily removed by brief immersion in chloroform, and the argentaffin layer is then uncovered; but once the cement has been deposited the removal of the wax becomes much more difficult.

In the *Tenebrio* adult the cement appears to come from a single type of gland only. Its contents will not reduce ammoniacal silver or stain in any other way. But if the insect is immersed for 5 min. in boiling chloroform, the contents of the glands stain black in the silver; the cement is regarded as a phenol-tanned protein associated in some way with lipide material. In the *Rhodnius* nymph there are two sorts of dermal glands (Wigglesworth, 1933). Type B is very numerous; it has a large cuticular vesicle filled with a watery solution of protein which does not give the argentaffin reaction, even after boiling in chloroform. Type A is much less numerous and its contents stain just like those of the glands in *Tenebrio*. It is suggested that the cement in the *Rhodnius* nymph consists of a mixture of these two secretions (Wigglesworth, 1948)—like the sclerotin of the cockroach öotheca (Pryor, 1940*a*).

The conspicuous segmental dermal glands in Lepidopterous larvae (Verson's glands) have commonly been regarded as the source of the moulting fluid (Verson, 1911). But Verson himself noted, and v. Buddenbrock (1930) confirms, that the vacuoles disappear from the glands at the very end of the moulting process, either immediately before or immediately after the moult. Zavrel (1935) observed the same thing in larvae of Chironomids, Blunck (1923) in *Dytiscus* and Poyarkoff (1910) in *Galeruca*. It therefore seemed probable that these glands also are producing a cement layer, and evidence that this is so has recently been obtained by Way (1948) working with the larva of *Diataraxia*. In this caterpillar the cement is poured out *before* the old skin is shed and, as might be expected, it varies considerably, and often rather irregularly, in thickness on different parts of the body. On the other hand, in the larva of *Sarcophaga* no dermal glands have been observed, and the entire cuticle appears to be the product of epidermal activity (Dennell, 1947*b*). But the deposition of the cuticle in the pre-exuvial stage of this larva has not been studied.

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* It is relevant to point out that the egg of the tick *Ornithodorus* is rendered waterproof by means of a waxy secretion poured out upon its surface by Géné's organ. This wax appears to be solubilized with protein. It is discharged through the pore canals of the cuticle covering the gland and not through any special ducts (Lees & Beament, 1948). Schmidt (1939) states that the wax of the abdominal glandular plates in *Apis* passes from the cells through the chitin.

Some authors in the past, such as Hass (1916) in *Gryllotalpa* and Schultze (1913) in the elytra of beetles, have suggested that the epicuticle (Grenzlamelle) of insects is the product of dermal glands, while Stegemann (1929) regards the Sekretschicht of Cicindelids as being a sort of greatly thickened Grenzlamelle. Poisson (1924) considered that the main function of the dermal glands in aquatic Hemiptera is to produce a secretion which renders the surface hydrofuge.

3. *Deposition of exocuticle and endocuticle*

In the later stages of moulting, while the formation of the epicuticle is being completed, the exocuticle (or pre-exuvial endocuticle) is laid down. By the time the old skin is shed this layer is complete and the parts which will become hard are already impregnated with phenols and perhaps with tyrosine (Wigglesworth, 1948). In the hours after moulting the polyphenol oxidase system is activated and hardening and darkening of the epicuticle and exocuticle take place. The elytra of *Carabus* remain white for about 3 hr.; blackening is complete in 18 hr. (Sprung, 1932). Meanwhile the inner endocuticle is being added and this may not be complete for 2 or 3 weeks (Sprung, 1932; Wigglesworth & Gillett, 1936). In the silkworm it is a much more rapid process. In the last larval stage, at 3 hr. after moulting, the cuticle thickness was as follows: exocuticle, $5{\cdot}3\,\mu$, endocuticle $4{\cdot}24\,\mu$; at 24 hr. it was: exocuticle $5{\cdot}3\,\mu$, endocuticle $18{\cdot}55\,\mu$ (Kuwana, 1933). Two-thirds or more of the cuticle is often added after moulting (Braun, 1875).

The lamination of the endocuticle has been attributed to rhythmic and synchronous periods of secretory activity by the cells (Drach, 1939; Zschorn, 1937), or to a slight shifting of the epidermal cells, the result of movements of the body, as successive layers of cuticle are deposited (Wigglesworth, 1933). But recently it has been shown by Picken *et al.* (1947) that both lamination and the orientation of chitin fibrillae in two preferred directions can occur in the chitinous cocoon of *Donacia* which is poured out as a viscid mass around the larva, quite detached from any cells. The very fine lamination visible with the electron microscope, which is due perhaps to differences in the packing of the micellae in successive layers, is likewise believed by Richards & Anderson (1942) to result from chemical changes in the cuticular substance after secretion.

The deposition of the 'Balkenlagen' of Coleoptera forms a special problem. Kölliker (1857) believed that the parallel strands were due to the splitting of an originally homogeneous layer, but their arrangement was regarded by Biedermann (1903) as too complicated for that. Kapzov (1911) describes their formation in *Cetonia* by the co-ordinated activity of the cells, which are wrapped round the sides of successive rods as they are deposited and then withdrawn before laying down the next sheet; these conclusions are supported by Korschelt (1923). Certainly the linear arrangement of the cells, for example in *Tenebrio*, during the deposition of the 'Balken' is very striking (Wigglesworth, 1948), and a similar relation between the line of the cell boundaries and that of the strands is described by Langner (1937) in Diplopoda; but it remains uncertain whether the cells impose their orientation on the cuticle. Picken (1940) suggests that orientation is due to mechanical forces acting during deposition. There are hints of an orientation of this kind in the cocoon of *Donacia* which is formed in the absence of any cells (Picken *et al.* 1947).

Although the formation of the epicuticle and the outer parts of the endocuticle in the third instar larva of *Sarcophaga* has not been studied, the subsequent changes in the growing larva and during the formation of the puparium have been followed in detail, and present some unusual features (Dennell, 1946). At the time the old skin of the second instar is shed, the new cuticle is 10μ thick. It increases to 40μ by the third day. That represents the 'outer endocuticle', the layer which contains the spiral pore canals already described (p. 413) and which eventually forms the sclerotized layer of the puparium. The inner endocuticle, which is devoid of pore canals, then begins to appear, so that at 3 days the outer endocuticle measures 40μ, the inner endocuticle 10μ. But as the inner endocuticle thickens the outer endocuticle also continues to grow, so that at 4 days the outer endocuticle measures 140–150μ, the inner 40μ. Just before pupation, the inner endocuticle has reached 80μ, about one-third of the total thickness. As the outer endocuticle thickens, the coiled pore canals become extended, and the laminae become more numerous. Since these laminae cannot result from the addition of new surface layers, Dennell suggests that the chitin-protein substance after incorporation separates spontaneously into sheets consisting perhaps of chitin-protein polymers separated by layers of pure chitin.

Little is known about the transfer of the reserves necessary for the building of the cuticle. Glycogen is moved to the integument of Crustacea while the new cuticle is being formed (Verne, 1924; Drach, 1939). In *Rhodnius* the massive reserves of glycogen and protein which collect in the fat-body before moulting are utilized in the formation of the cuticle. Glycogen is mobilized just before the chitinous endocuticle is laid down; it appears in the epidermal cells in the form of large deposits at the time of moulting, when these cells are most actively engaged in chitin secretion, and it disappears when the main period of chitin formation is past. The deposits of protein in the fat-body show changes more or less parallel with those of glycogen; they, too, form visible masses in the epidermal cells (Wigglesworth, 1947*a*). There is little justification for the view of Bounoure (1919) that chitin is to be regarded as an excretory product.

The deposition of the cuticle is naturally influenced by nutrition. The cuticle of the fifth instar silkworm larva, at the second day after moulting, contains 6·0 mg. of chitin. After feeding for two more days it increases to 13·7 mg.; but if the larva is starved, it reaches only 7·6 mg. by that time (Kuwana, 1933). In the *Rhodnius* nymph the thickness of the cuticle depends upon the size of the blood meal which was taken before moulting. If 32 mg. of blood were taken, the cuticle was about 8μ thick; if 140 mg. were taken, the thickness was about 20μ; and if an insect with a very thin cuticle was given a series of small meals after moulting (not large enough to initiate a new moult), the cuticle gradually increased in thickness (Wigglesworth, 1942*a*).

4. *Function of the pore canals*

We are now in a position to consider the function of the pore canals. Whatever their fate in the fully hardened cuticle, and we have seen that in some insects their contents become converted into cuticular substance (p. 413), there is no doubt that during the deposition of the cuticle the pore canals contain cytoplasmic filaments, and in many insects this state persists. It was suggested by Leydig (1860) and by Braun (1875) in *Astacus*, by Holmgren (1902*a*) in the oviduct of *Calliphora*, by Verne (1921) in various Crustacea and by Poisson (1924) in aquatic Hemiptera, that chitin in fluid or semi-fluid form is deposited around filiform outgrowths from the epidermal cells and that those outgrowths later constitute the pore canals. As has often been pointed out, this mechanism recalls the formation of dentine by the odontoblasts of vertebrates (Hass, 1916).

But the pore canals are not essential for the deposition of chitin. In the adult *Tenebrio* the pore canals converge as they approach the epidermal cells, so that in the fully formed cuticle there are intervening areas of the endocuticle which are devoid of pore canals (Wigglesworth, 1948). In the enormous growth of the endocuticle which takes place in the engorged female tick *Ixodes*, the pore canals become very widely separated, but it is difficult to prove whether or not, as Nordenskiöld (1908) supposes, the pore canals are concerned as intermediaries in this growth (Lees, 1948). In the larva of *Sarcophaga*, the inner endocuticle is devoid of pore canals, and the outer endocuticle continues to grow by the addition of cuticular substance diffusing through the inner endocuticle in spite of the fact that the pore canal contents are completely chitinized. Here the pore canals can play no part (Dennell, 1946).

It seems more likely that the true function of the pore canals is to enable the epidermal cells to act at a distance upon the superficial layers of the cuticle (Wigglesworth, 1933). We have seen that they pour out the 'polyphenol layer' over the surface of the cuticulin, and later probably secrete the wax, both during moulting (p. 436) and for the repair of abrasions (p. 443). The control of oxidations in the hardening cuticle is probably effected through these canals. Whether the pore canals function in this way in the formation of the epicuticle in the larva of *Sarcophaga* has not been investigated, but in the later stages of this insect, when the puparium is formed, the pore canals are already filled by chitinous filaments and both enzymes and substrates apparently diffuse outwards through the general substance of the cuticle, and there is no evidence that the pore canals function as conducting channels (Dennell, 1947*a*).

5. *Formation of bristles and scales*

The spicules or microtrichia on the cuticle arise as cytoplasmic outgrowths, apparently containing a pore canal, and are often composed solely of epicuticular material, cuticulin covered with cement (Kühnelt, 1928*c*; Richards & Korda, 1947). The articulated bristles (Hufnagel, 1918; Haffer, 1921) and scales (Reichelt, 1925; Stossberg, 1938) are formed by two cells. The primary cell, called the trichogen cell by Graber (1877), gives off a process which in due course becomes the hair or scale. The secondary cell or tormogen (Wigglesworth, 1933) grows around this process in the form of a collar (Lees & Waddington, 1942; Schwenk, 1947) and forms the socket.

The growth and form of these bristles is very largely influenced by the fine structure and properties of the material of which they are composed (Lees & Picken, 1945). The bristles of *Drosophila*, as we have seen (pp. 415, 417), are made up of micelles orientated in the long axis. It is not possible to say what determines the initial orientation of the anisometric elements at the surface of the developing rudiment, but growth takes place chiefly at the tip; perhaps in this region the free ends of the chitin chains are constantly added to. The trichogen cell grows enormously (it may increase 1000 times in volume), and it is suggested that the pressure set up in the trichogen as the result of this volume increase is responsible for the outgrowth of the process. Perhaps a bristle may be regarded as an object blown in plastic material—a long-chain high polymer in the rubber-like state, almost devoid of cross-linkages between chains. As the wall substance ages it hardens; this decline in plasticity occurs progressively from the moment the wall is formed and apparently precedes the process of tanning (cf. the hardening of the epicuticle (p. 427), where it is suggested that denaturation of the protein and perhaps polymerization of the lipides first occur). A normal bristle is produced only if there is a nice balance between increase in cytoplasmic content and synthesis of material in the wall. The various mutant bristle types in *Drosophila* may be explained by upsets in these orderly processes, such as disturbances in the ratio of volume increase and area increase, or abnormalities in the properties of the bristle wall and so on.

Very elaborate changes take place in the formation of scales, although in principle the mechanism of development is the same as that of hairs and bristles (Reichelt, 1925; Stossberg, 1938). The scale is the complicated cell wall of an exceedingly enlarged epithelial cell. The formative cell gives out a process which rapidly becomes club-shaped, flattens, and increases in length and width. Finally, the plasma of the formative cell breaks down and air penetrates through openings in the cuticular wall into the interior of the scale (Kühn & An, 1946). Recent work by Picken (1948) suggests that the flattening of the scale may be due to the rudiment with a circular cross-section becoming mechanically unstable when its diameter exceeds a certain limiting value, the force operating being in part, perhaps, the adhesion of the rudiment to the surface of the wing. It is further suggested that the longitudinal ridges and the cross-connexions between them may be due to periodic protein-polysaccharide synthesis analogous to periodic crystallization *in vitro*, the protein-polysaccharide material being thus incorporated into the substance of the scale as it expands.

6. *Digestion of the old cuticle, the moulting fluid*

As development proceeds the space between the old cuticle and the new becomes filled with a more or less copious fluid, the moulting fluid. Most authors have regarded this primarily as a lubricant to facilitate moulting. Yet most insects are almost dry at the time the skin is cast, whereas if they are dissected a day or two before moulting, there is abundant fluid present. The main function of the moulting fluid is probably accomplished therefore before ecdysis actually occurs.

That function is doubtless the digestion and solution of the inner layers of the old cuticle (Plotnikov, 1904; Tower, 1906). It is a neutral salt-free fluid with proteins in solution which contains a protease and probably a chitinase (Wigglesworth, 1933). It attacks only the endocuticle, which as a rule is completely broken down; the exocuticle and epicuticle are not affected. Along the various 'ecdysial lines' or 'lines of weakness' in the cuticle, usually on the head and thorax, an exocuticle is wanting, so that when the endocuticle is dissolved, the cuticle breaks on the slightest pressure and the insect can escape from the old skin (Plotnikov, 1904; Tower, 1906; Eidmann, 1924). By this process 86·5% of the abdominal cuticle in *Rhodnius* is absorbed (Wigglesworth, 1933); 83–85% in the larva of *Tenebrio* (Evans, 1938), and 80–90% in the silkworm, in which the exuviae contain only 10–20% of the chitin present before moulting (Kuwana, 1933; Bergmann, 1938).

The moulting fluid is generally regarded as coming from the dermal moulting glands (Plotnikov, 1904; Tower, 1906; Schultze, 1912; Woods, 1929, etc.). On the other hand, v. Buddenbrock (1930) in Lepidoptera, Blunck (1923) in *Dytiscus* and Poisson (1924) in aquatic Hemiptera, regard the fluid as being mainly a product of the epidermal cells. In studying moulting in *Rhodnius* (Wigglesworth, 1933) the general view, that the dermal glands were secreting the moulting fluid, was adopted; for it was argued that the epidermis could not be secreting the digestive enzymes (as Tower (1906) had claimed), because digestion does not begin until a day or so before moulting, by which time the cells are covered by a thick layer of new cuticle.

But more recent work (Wigglesworth, 1947*a*) has led to a revision of these views. The dermal glands reach the height of their activity at the very end of the moulting period, and discharge their secretion, which forms the cement layer (p. 436), after the skin is shed. It is possible, of course, that at an earlier stage they may be secreting the digestive enzymes. But the argument that these cannot come from the epidermal cells because they are covered by cuticle falls to the ground, since it has been shown that the cells are in fact discharging the polyphenol secretion and the waxes upon the surface of the cuticle almost up to the time of moulting.

It is certain that the epidermal cells *can* digest the cuticle in the absence of dermal glands, for this happens when isolated patches of the integument in *Ephestia* undergo moulting (Kühn & Piepho, 1938); the endocuticle of the ptilinum in adult *Calliphora* is completely digested and absorbed, being reduced from 25μ to about 1μ during the 2 weeks after the fly has emerged (Laing, 1935); and in the engorged female tick *Ixodes*, the cuticle decreases progressively in thickness from the time egg-laying begins (Ruser, 1933). The products of digestion are absorbed through the general surface of the cuticle; dyes such as neutral red and indigo-carmine can be absorbed at the same time by this route (Wigglesworth, 1933).

VI. THE CUTICLE AS A LIVING SYSTEM

The cells of the epidermis must be the most versatile in the insect body. Apart from their co-ordinated movements and growth which control the form of the organism, they fulfil a series of complex functions in the actual deposition of the cuticle. The epidermal cell secretes the cuticulin of the epicuticle, the chito-protein of the inner layers, perhaps the chitinase and protease which dissolve the old skin, then the polyphenol-containing material, then the wax and finally the oxidase which completes the hardening of the cuticle. All these activities are nicely timed and synchronized throughout the body.

These processes are probably controlled immediately by humoral means, and more remotely perhaps by the nervous system. In the adult blowfly *Calliphora*, hardening and darkening normally occur within 30 min. after emergence, but they may be delayed for 7 hr. at least if the insect is obliged to continue digging through the soil (Fraenkel, 1935). A similar delay occurs in the tracheal system of mosquito larvae as the result of enforced submergence (Wigglesworth, 1938*a*). Hardening in the cuticle may take place anywhere if the epidermal cells below die from any cause. The active substance bringing about the change circulates freely in the blood at the time of pupation. Initiation of hardening is correlated with the humoral changes which bring about pupation and metamorphosis (Becker, 1941).

There is no question that epidermis and cuticle remain very much alive, right to the surface, up to the time of moulting. In the fully hardened insect it is customary to regard the cuticle as a dead structure. But even in the wings and elytra the epidermis persists, and to that extent the wings remain alive (Reichelt, 1925). Even in adult insects, injuries to the cuticle are made good and new cuticle is laid down (Wigglesworth, 1937).

To what extent the cuticle itself remains a living tissue is not so sure. In the sclerotized cuticle of the abdominal sternites in *Tenebrio* the outer parts of the pore canal appear to be filled with sclerotized contents, and it is difficult to believe that any living tissue remains (Wigglesworth, 1948). But if insects are immersed fresh in liquid paraffin, for example, small droplets of water slowly exude from the surface, even of the hard parts and along the bristles. These droplets pass out much more readily through the cuticle of young insects (Wigglesworth, 1942*a*).

In many insects the pore canals apparently always retain cytoplasmic contents extending up to or through the cuticulin layer and separated from the exterior only by the wax layer and the cement. We have seen that abrasion of these superficial layers with fine mineral dusts, which cause no visible injury to the cuticulin layer, renders the cuticle permeable to water. Although the bodies of the epidermal cells are separated from the point of injury by cuticle some 20–30μ thick, they react as though they had been wounded, collect around the site of injury and secrete fresh wax through the pore canals to restore the waterproofing of the cuticle (Wigglesworth, 1945).

This wax is not covered by cement, so that it is readily removed by brief extraction with chloroform. It is worth noting that while it is being secreted, the wax, even the hard wax of *Rhodnius*, can be taken up by adsorption on to dusts (Wigglesworth, 1945); and in the tick *Ornithodorus*, in which the same process of repair occurs, the growth habit of the wax deposits exuded is greatly influenced by the humidity of the air (Lees, 1947). This affords evidence that the wax is secreted in an aqueous medium. Where recovery from such superficial injury occurs, it is impossible not to regard the cuticle as a living tissue.

The integument also shows some vital activity in the uptake of water from the environment (p. 432). Desiccated ticks (*Ixodes*) in contact with liquid water, take up as much fluid as they need and then cease. They are able to take up water vapour from the air at a relative humidity as low as 92%. This activity has been proved to reside in the integument. Presumably the substance of the cuticle acts as a hygroscopic material from which water is subsequently removed by secretory activity on the part of the epidermal cells. At humidities as low as 50% the secretory activity of the epidermis is also concerned in restricting the transpiration of water, for the tick killed with ammonia loses water at a more rapid rate than the living tick; this active secretory force is added to the passive waterproofing provided by the wax layer. It is interesting to note that although the different species of ticks from progressively drier environments are progressively more effectively waterproofed, the lipide materials having higher transition points the drier the natural environment (as in insects (p. 429)), all can absorb water vapour from the humid air with about equal rapidity (Lees, 1946). Abrasion with fine dust over a limited area of the cuticle inhibits the secretory uptake of water by the tick, even in saturated air, but recovery takes place within a day or so (Lees, 1947).

The same phenomenon is seen in insects: *Tenebrio* larvae can take up water at 88% R.H. (Mellanby, 1932), the grasshopper *Chortophaga* at 82% (Ludwig, 1937) and the prepupating larva of the flea *Xenopsylla* at humidities as low as 45% (Edney, 1947). But it has not yet been proved in the insect that the general integument is responsible.

One must conclude that the integument continues to play an active part, not only in the maintenance of its own integrity, but in the exchanges which take place through it. In many arthropods the cuticle is very much alive.

VII. SUMMARY

The cuticle consists of a relatively soft and colourless *endocuticle*, hardened and darkened in its outer part in some places to form a rigid *exocuticle*, and a complex *epicuticle* made up of several layers.

The *endocuticle* consists of *polyacetylglucosamine* (*chitin*) intimately associated with a characteristic *protein* (*arthropodin*). Perhaps these are combined in the form of a mucoprotein in which the relative amounts of protein and polysaccharide vary with the type of animal and with the part of the body. The substance of the endocuticle separates, apparently spontaneously, into laminae of varying dimensions.

In most insects the submicroscopic crystallites of chitin, and perhaps protein, tend to lie at random with the long axis in the plane of the cuticle. They can be orientated by tension or compression. In the Coleoptera they are arranged in microscopic bundles ('Balken') lying parallel in a given lamina, but at an angle of 60° in successive laminae. The crystallites are orientated also in the long axis of tendons and bristles.

In the *exocuticle* the protein is tanned by quinones derived by oxidation from dihydroxyphenols. This *tanned protein* (*sclerotin*) impregnates the chitin framework to form a rigid fabric (like cellulose impregnated with a resin plastic) which is moderately impermeable to water. Hard cuticles in insects are always dark, largely because the quinonoid groups are chromophore, in part perhaps because during the oxidative hardening some true melanin is formed from tyrosine. In a few insects impregnation with lime takes the place of tanning.

The cuticular substance has a tendency to crystallize in the form of multiple thin plates; these are responsible for the iridescent colours of many insects.

The *epicuticle* is responsible for most of the impermeability to water. It consists of a thin layer of *tanned lipoprotein* (*cuticulin*), a layer of *crystalline waxes* about a quarter of a micron thick, and a layer of *cement* (likewise consisting, perhaps, of tanned protein containing some lipides) protecting the wax.

The wax varies in character from a soft grease to hard white crystalline materials. If the temperature is raised to a critical level, some 5–10° C. below the melting-point of these waxes, the insect shows a sudden increase in the rate of transpiration. If the cement and wax layer are abraded by fine dusts or removed by lipid solvents, the loss of water increases enormously. The properties of the epicuticular layers control to some extent the entry of insecticides through the cuticle.

In the *deposition of the cuticle* the cuticulin layer is first laid down; the lipoproteins which compose it appear to come from the oenocytes. Formation of the endocuticle takes place around cytoplasmic filaments (the *pore canals*) which extend from the interior of the epidermal cells and appear to penetrate the cuticulin layer of the epicuticle. Droplets of material rich in polyphenols are exuded from the tips of the pore canals and fuse to form a continuous layer over the cuticulin. The wax is then secreted, during the last few hours before *moulting*, and covers the polyphenols. Almost immediately after moulting (in some insects before moulting) the cement is poured out from the dermal glands.

The inner layers of the old cuticle are digested by enzymes contained in the moulting fluid and probably secreted by the general epidermis. The products are absorbed, together with almost all the fluid, before moulting occurs.

Hardening and darkening take place after moulting as the result of the activities of the oxidative enzymes in the cuticle which convert the dihydroxyphenols into quinones.

The function of the pore canals is probably to enable the epidermal cells to act at a distance upon the superficial layers of the cuticle. In some insects they still contain cytoplasmic filaments in the fully formed cuticle; in others their contents are converted into chitin, into sclerotin, or a mixture of the two.

The greater part of the endocuticle is laid down after moulting.

The *integument* of many insects remains *alive*. Removal of the wax and cement layers by gentle abrasion is repaired by the secretion of fresh wax. And in some forms water can be actively absorbed from the atmosphere even when this is far from being saturated with moisture.

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ANTIBIOTICS*

By SELMAN A. WAKSMAN
N.J. Agricultural Experiment Station, Rutgers University, New Brunswick, U.S.A.

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I. INTRODUCTORY

Although the phenomenon of antagonism or antibiosis among micro-organisms has been recognized since the early days of bacteriology, and the ability of various bacteria and fungi to produce antibacterial substances has been well appreciated (Waksman, 1937, 1941, 1947*a*), the real development of the subject of antibiotics is of very recent origin. Only a decade ago antibiotics were usually spoken of as lysins, bacteriolysins, bacteriotoxins, bactericidal substances, bacteriolytic agents, antibacterial agents, antagonistic substances, lethal principles, mould toxins, staling principles, and many others. Formation of such substances by micro-organisms, if recognized, was considered at best of limited application, and at worst as microbiological curiosities.

This is true of the various antibacterial substances of microbial origin that were isolated or merely recognized before 1938, as shown by such products of bacteria as pyocyanase and pyocyanin, by various agents produced by fungi which included penicillin and gliotoxin, and by two substances of actinomyces origin, namely, actinomycetin and actinomyces lysozyme. Recognition of the important chemotherapeutic potentialities of these microbial products was very limited. Because of their selective action upon different bacteria they were utilized for separating bacterial cultures from one another. The ability of some to bring about the lysis of certain bacterial cells was taken advantage of in the preparation of vaccines (mycolysates). Many efforts were made to find agents that might have an effect upon phytopathogenic fungi or bacteria. The various attempts to utilize cultures of micro-organisms, their cell constituents, or their metabolic products for treatment of infections were either complete failures or yielded results that were never sufficiently substantiated.

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The very nature of the antimicrobial agents produced by micro-organisms and their significance in the growth and nutrition of the organisms producing them, on the one hand, and of the organisms upon which they were active, on the other, were highly confused. It is sufficient to cite, in this connexion, the ideas on this subject expressed by Papacostas & Gaté in 1928 in their comprehensive treatise, *Microbial Associations and their Therapeutic Applications.* These investigators believed that the various interactions among bacteria in mixed cultures *in vitro*, designated as 'antibiosis', were different from the interactions among bacteria in mixed infections *in vivo*, designated as 'antagonism'. Even such clear-cut investigations as those of Fleming (1929) and Weindling (1934) on penicillin and gliotoxin produced by fungi, or of the many students concerning the antibacterial substances of spore-forming bacteria, as well as the numerous studies of pyocyanase carried out for nearly half a century, did not fit into a well-recognized system and received, therefore, only scanty consideration.

This lack of recognition of the importance of antibiotics as potential chemotherapeutic agents was emphasized by myself in a recent address (1947*c*), from which one may quote: 'Just seven years ago to-day, the present speaker presided at a conference that may become known as the first antibiotics conference ever held in this country or in any other country. This took place in the form of a round-table discussion arranged during the meetings of the Society of American Bacteriologists held in St Louis, at the end of December 1940. This round-table had to do with a discussion of our knowledge of the production by micro-organisms of antibacterial or bacteriostatic and bactericidal substances, the word antibiotic in the present accepted meaning not having been recognized as yet. The organizer of the round-table, Dr Dubos, and the chairman found it difficult to obtain, for this brief two-hour conference, enough contributors who had sufficient information about the subject to participate in the discussion, or who were willing to hazard a guess as to its future potentialities. Only five years later, namely, in January 1946, the New York Academy of Sciences held the first comprehensive three-day conference on the subject of antibiotics. The attendance was about 500, representing the various fields of microbiology, chemistry, medicine and industry. The comprehensive programs and the well-attended meetings bore ample evidence to the fact that in this brief period of only five years, a new branch of science was born. The program of the present meetings may well serve as evidence that this new branch of science, namely, that of antibiotics, is still in a growing stage and has not yet reached full maturity. New agents are constantly being discovered. Several antibiotics have already found important applications as chemotherapeutic agents. To their exploitation, great industries have devoted considerable time and energy. Many diseases that could not be combated previously have been found to lend themselves readily to therapy. Old methods of treatment of numerous important diseases have been replaced or supplemented by new ones, frequently simpler in nature, cheaper in cost, and more certain in results.'

In the period of seven years, cited above, more than a hundred different antibiotics have been isolated and described; hundreds of thousands of cultures representing different groups of micro-organisms have been isolated from various substrates, and tested for their ability to inhibit the growth of bacteria and other micro-organisms, and to produce antibiotic substances. The literature of penicillin has expanded from four or five papers, known in 1939, to many thousands of papers as well as several volumes written in many different languages, in 1947. The subject of streptomycin, the isolation of which was announced only a little over four years ago, has already a voluminous literature covered by more than 1000 papers. Other antibiotics, notably tyrothricin, subtilin, and bacitracin, have received their share of attention, and still others, such as licheniformin, nisin, chromomycetin, aerosporin and polymyxin, are still too recent to warrant definite conclusions.

In a recent review of the subject, Florey (1947) listed thirty or more antibiotics produced by fungi. Some twenty-five antibacterial agents are now known to be formed by actinomycetes. Numerous substances have been isolated from cultures of bacteria. Any attempt, therefore, to summarize a subject which has grown with such tremendous strides in less than a decade can at best be only schematic and general in outline.

In a recent editorial entitled 'The antibiotic age', Tillett (1948) wrote: 'It has been an exciting era that has seen a drop in the mortality....Since bacterial infections of one kind or another make up such a considerable proportion of the organic ills of man, it is readily understandable that the practical uses of chemo- and antibiotic therapy should be explored to the greatest degree, and that published reports of their successes and limitations should fill medical journals in the most conspicuous fashion....The veering of emphasis which has resulted from antibiotic therapy may be seen in surgical management in which patients are well saturated with antibacterial reagents before, during and after operative procedures. Since the hazards of infection are either eliminated entirely or rendered less likely, interest now centers around elements in technic that promote the maintenance of normal *functional activity in spite of surgical alterations, and in minimizing the cellular and* tissue damage that is an unavoidable part of surgical incisions and visceral manipulation....In viewing the antibiotic age, perhaps greater interest, even if of a more speculative nature, centers around whether or not the present-day satisfaction with the success of antibacterial therapy can be transformed into a permanent optimism for generations to come.'

II. NATURE OF ANTIBIOTICS

Antibiotics have been defined (Waksman, 1947*b*) as chemical substances produced by micro-organisms which have the capacity to inhibit the growth of and even to destroy bacteria and other micro-organisms. Antibiotics are characterized by certain properties which distinguish them sharply from the ordinary chemical antiseptics and disinfectants. In order to understand the nature, mode of action and potential utilization of antibiotics, it is essential to have a clear picture of their origin, chemical composition, physical properties and biological activities, both *in vitro* and *in vivo*. These characteristics can be briefly summarized as follows:

(1) Antibiotics are produced primarily by micro-organisms, which are designated as 'antagonists', since they have the property of antagonizing or inhibiting the growth of other micro-organisms. Some antibiotics are readily secreted into the culture medium, whereas others are retained largely in the microbial cells. Higher plants and animals also produce substances which possess antimicrobial properties comparable to those of true antibiotics; this is true of the plant phytoncides and of the animal lysozyme and erythrin. It is best to consider them, however, as antibiotic-like in nature, otherwise compounds such as quinine would also be classified with the antibiotics.

(2) One of the most distinguishing properties of antibiotics is their selective action upon bacteria and other micro-organisms. Some are active largely upon Gram-positive and only upon very few Gram-negative bacteria, whereas others affect alike various bacteria within each of these groups. Some act upon fungi, whereas others do not. Some are also active against rickettsiae. Most antibiotics have very little if any effect upon viruses. The differences in the antimicrobial action of antibiotics are both qualitative and quantitative in nature. One may thus speak of a bacteriostatic or an antibiotic spectrum, to designate the range of selective antimicrobial action of a given substance upon a number of representative bacteria and other micro-organisms.

(3) Antibiotics vary greatly in their physical and chemical properties. A large number of chemical compounds which possess antibiotic properties have now been isolated. These may either belong to distinctly different groups of chemical compounds, or they may show close similarity to one another. The latter is true, for example, of the different penicillins, produced by the various fungi under different conditions of culture, or of the different forms of streptothricin, which may be produced by different strains of certain chromogenic actinomycetes under different conditions of cultivation.

(4) Certain micro-organisms are capable of producing more than one antibiotic. *Pseudomonas aeruginosa* has been reported recently to form, in addition to the older pyocyanase and pyocyanin, also a group of pyo-compounds, pyolipic acid and certain other substances. *Bacillus brevis* produces the tyrothricin complex, from which gramicidin and tyrocidine have been isolated; the exact relation to these of gramicidin S is still not well established. *Penicillium notatum* and *P. chrysogenum* produce not only different types of penicillin, but also a different antibiotic, designated as 'penatin', 'penicillin B' or 'notatin'; *Aspergillus flavus* produces aspergillic acid and penicillin, the latter having been described under a number of different names. *A. fumigatus* produces spinulosin, fumigatin, fumigacin and gliotoxin; whether the substances produced by this organism and found to be active against *Mycobacterium tuberculosis* or tumour tissues are identical with any of these

still remains to be determined. *Streptomyces griseus* produces two forms of streptomycin, actidione, and an antibiotic present in the mycelium of the organism. Various strains of *S. lavendulae* were found to produce a number of antibiotics, which have been isolated and described, namely, streptothricin, lavendulin, streptolin and chloromycetin. It is no wonder, then, that an isolated antibiotic may differ in its antibiotic spectrum from the metabolite solution from which it has been isolated. The culture filtrates of *Penicillium notatum* and *Streptomyces griseus* are active against certain bacterial toxins (antidotic properties), whereas the purified antibiotics produced by these organisms, namely penicillin and streptomycin, do not possess that activity.

(5) Some antibiotics are produced by more than one organism. Penicillin is formed by numerous strains of *Penicillium notatum, P. chrysogenum* and *Aspergillus flavus*, and by a variety of other fungi. Gliotoxin is produced by certain strains of *Trichoderma viride, Gliocladium* sp., *Aspergillus fumigatus* and certain other fungi. Clavacin, an antibiotic which has also been designated as 'claviformin', 'patulin', 'clavatin', and 'expansin', is formed by numerous fungi, including *Aspergillus clavatus, Penicillium claviforme, P. patulum, P. expansum, Gymnoascus* and many others. Actinomycin is produced by different actinomycetes belonging to the genus *Streptomyces*. Streptomycin is produced by *S. griseus* and *S. bikiniensis*; streptomycin-like substances are produced alone, or in mixture with streptothricin and possibly with other antibiotics, by a variety of other *Streptomyces* species; this is true, for example, of antibiotic F. Since antibiotics are usually named after the organism producing them, considerable confusion has frequently resulted from the duplication of names for the same antibiotic, which has been studied in crude preparations isolated from different organisms.

(6) The effect of the medium or of the substrate upon the antibacterial activities of different antibiotics varies greatly. Some substrates have no effect at all upon the antibacterial activities of a given antibiotic; others may reduce considerably such activity by neutralizing the antibiotic action by some constituent of the medium, such as peptone, glucose, serum or salt. Some substrates, like blood, may adsorb the antibiotic and completely inactivate it. Certain antibiotics may, therefore, show marked differences in their *in vitro* v. *in vivo* activities.

(7) Antibiotics are affected differently by micro-organisms; some, like penicillin, are rapidly destroyed by a variety of bacteria, whereas others, like streptomycin, are highly resistant to the microbial action.

(8) The mechanism of antibacterial action of one antibiotic is distinct from that of another. Some antibiotics interfere with the growth of micro-organisms and with their cell division; some influence microbial respiration; others affect the utilization of essential metabolites. Although antibiotics are often spoken of as primarily bacteriostatic agents, their bactericidal properties may also be very pronounced; the nature of the bacteria affected, their stage of growth, and the composition of medium or substrate are of great importance in this connexion. These phenomena are particularly significant from a chemotherapeutic point of view, because of

differences in the action of antibiotics upon the cells of bacteria and upon those of body tissues.

(9) Antibiotics vary greatly in their toxicity to animals. Some, like actinomycin, are extremely toxic; others, like penicillin, have virtually no toxicity at all; most others fall between these two extremes. The nature of the toxic effect depends upon the antibiotic and upon the animal.

(10) Different strains of a given bacterial species vary greatly in their sensitivity to a given antibiotic. Sensitive organisms can gradually develop resistance to certain antibiotics if allowed to be in contact with them for any length of time. Different antibiotics vary greatly, however, in the mechanism with which such resistance develops. Some like streptomycin allow rapid development of resistance against most bacteria originally sensitive to it, whereas others, like penicillin, allow only gradual development of resistance by very few sensitive bacteria. The loss of resistance also differs with the antibiotic and the sensitive bacteria.

(11) Because of these differences, antibiotics vary greatly in their chemotherapeutic potentialities. Although more than one hundred antibiotics have now been isolated and described, only very few of them have so far found chemotherapeutic application.

III. BACKGROUND OF ANTIBIOTICS

1. *Early observations*

The remarkable developments in recent years in the field of antibiotics, which have given such a great impetus to the progress of chemotherapy, can be traced to several distinct sources, resulting from investigations in various fields of science.

(*a*) Since the early days of bacteriology, during the latter part of last century, the fact was recognized that certain microbes are capable of repressing, and even of destroying, in culture, various other microbes including both pathogenic and saprophytic forms.

(*b*) Mixed infections were found to behave differently from infections caused by single organisms; some of the organisms in such mixed infections repressed the growth of the other organisms. An attempt was made to treat infections by less pathogenic organisms, as in the treatment of anthrax with streptococci or with *Pseudomonas aeruginosa*; frequently saprophytes were used for treating infections, as in the early attempts by Cantani to treat tuberculosis with *Bacterium termo*.

(*c*) Early studies of the microbiological population of the soil brought out the fact that the great majority of disease-producing organisms which find their way into the soil, in the form of excreta from infected individuals or in the very bodies of such individuals, gradually disappear in the soil. This was found to be due largely to the presence in the soil of microbes, known as antagonists, which bring about the destruction of the disease-producing organisms.

(*d*) Certain organisms isolated from the soil or from dust and grown in artificial media were found to produce substances, later designated as antibiotics, which had the capacity to inhibit bacterial growth. These substances, such as pyocyanase and penicillin, appeared to be the mechanisms which were responsible for the disappearance of bacteria that were in contact with the antagonistic organisms.

(*e*) Various attempts were made to utilize some of the above substances for chemotherapeutic purposes; however, the results obtained were largely inconclusive. This was due, partly at least, to an insufficient differentiation between the activities of the living organisms or the antagonists, and of their chemical products, which possessed antibacterial properties, or the antibiotics. The fact was established, however, that these substances are highly specific, being effective only against certain organisms and not against others.

The above observations and certain others bearing upon the interrelationships among micro-organisms were substantiated by numerous experiments. Many of these were only cursory and unco-ordinated in nature. The experimental evidence that was submitted in support of these observations did not exert any profound influence upon the future developments of the subject or upon the course of medical practice. Although many of these investigations were fundamental in nature, the results did not seem to fit into a well-co-ordinated pattern. They certainly did not point to the great potentialities in the field of utilization of antibiotics for disease control as it is visualized to-day. Only a few illustrations can be cited here. For a more complete survey of the literature, the reader is referred to the second edition of the book on *Microbial Antagonisms and Antibiotic Substances* (Waksman, 1947*a*).

The modern period of antibiotics may be said to date back to 1939. At that time it became definitely recognized that certain micro-organisms, including various bacteria, fungi and actinomycetes, have a marked capacity to inhibit the growth of bacteria and other micro-organisms. These organisms were shown to produce, under certain conditions of nutrition, antibacterial substances which were able to inhibit the growth of and even to destroy other organisms. Mention should be made of such substances as penicillic acid, penicillin and gliotoxin as products of fungi; pyocyanase, pyocyanin, lysogenic compounds, and many others as products of aerobic bacteria; lysozyme-like and enzyme-like substances as products of actinomycetes. It was well established at that time that these compounds varied in chemical nature, in selective action upon different micro-organisms, and in toxicity to animals.

2. *Chemotherapeutic experiments*

Numerous attempts were made, previous to 1939, to utilize antagonistic micro-organisms and their metabolic products for combating infections (Florey, 1946; D'Arcy Hart, 1946; Waksman, 1947*a*, 1947*d*). These can be summarized briefly in the following historical sequence:

(1) Soon after Pasteur demonstrated, in 1877, that the development of pathogenic organisms could be repressed by the presence of non-pathogens, various efforts, largely unsuccessful, were made to replace the pathogens by other microbes.

(2) Non-pathogenic organisms were used for immunizing the human and animal body, in order to protect it against pathogens.

(3) Microbial products were utilized for topical and parenteral administration against various localized or generalized infections.

Cantani was the first to attempt, in 1885, the practical utilization of certain common bacteria for the treatment of patients suffering from tuberculosis. Cultures of a saprophytic organism of doubtful purity were blown into the lungs of the patient. This treatment resulted in a certain improvement in the patient's condition and was accompanied by the appearance of the saprophyte in the sputum. In 1887, Emmerich demonstrated that the injection of streptococci into animals enabled them to withstand infection with *Bacillus anthracis*. In 1889, Bouchard showed that the inoculation of animals with *Pseudomonas aeruginosa* or the pyocyaneus organism also gave protection against anthrax.

The replacement of pathogenic bacteria causing infections by saprophytic organisms or by potentially less pathogenic bacteria forms a most interesting chapter in the history of microbiology and medical practice. The introduction into the human intestines of harmless lactic acid organisms to replace potentially dangerous enteric bacteria, a form of therapy first initiated by Metchnikov, has found wide application. The use of *Escherichia coli* for the purpose of replacing pathogenic bacteria in the gut was first postulated by Nissle in 1916; this as well had a certain vogue. In the treatment of diphtheria, recourse was had to the use of a variety of bacteria, ranging from lactic acid organisms to *Staphylococcus aureus*; the resulting effects of the antagonist have not always been too favourable to the host, as one might expect from an excessive application of *S. aureus*. Use of various forms of yeasts, by various methods of administration, has also had a certain popularity, with rather uncertain results. Cultures of lactobacilli were used not only for internal but also for external administration, as in the treatment of vaginal trichomoniasis. The application of *Lactobacillus acidophilus* cultures was also said to be beneficial in the treatment of various forms of diarrhoea and dysentery.

Emmerich & Löw, in 1899, used a preparation of *Pseudomonas aeruginosa*, designated as 'pyocyanase', for combating infections. This may be considered as the first attempt to utilize an antibiotic substance for chemotherapeutic purposes. This was followed by numerous other efforts of a similar nature. The results obtained were rather uncertain and often disappointing, in spite of certain favourable indications at other times. Pyocyanase preparations appeared to find special application in surface therapy. They were used clinically in a number of infections, including nasal sinuses, Vincent's angina, diphtheria, and streptococcal mastitis in mammalian animals.

Fungus products, as well, were tried for their clinical potentialities. Vaudremer, for example, in 1913 used certain metabolic products of *Aspergillus fumigatus* for combating tuberculosis; although some 200 patients were thus treated, the results were rather inconclusive. Gratia used cultures of certain actinomycetes for the purpose of lysing typhoid bacteria; the preparations thus obtained were designated as mycolysates and used for immunizing purposes.

A number of other preparations were obtained from cultures of bacteria and fungi; frequently, the total culture filtrate of the organisms was used. These preparations were occasionally found to possess marked antibacterial properties. Some of them were active not only *in vitro*, but also *in vivo*. A few of them were used clinically in the treatment of human infections, such as anthrax, diphtheria and tuberculosis, with varying degrees of success. No broad generalization could be made, however, on the basis of these experiments concerning the possibility of utilizing products of micro-organisms as chemotherapeutic agents, except under certain conditions.

3. *A new approach*

To bring all these observations together into one system, and thus lay the groundwork for the science of antibiotics, and especially their potentialities as chemotherapeutic agents, a synthesis was needed. This required the co-ordinated efforts of the microbiologist, the chemist, the pharmacologist and the clinician, in order to test various micro-organisms obtained from different substrates for their ability to inhibit bacterial growth and produce antibiotic substances, isolate such substances from the metabolite solution, evaluate their toxicity and effectiveness in the animal body, and finally test them clinically. This was brought about in 1939–40, when the isolation of tyrothricin, soon followed by the reisolation of penicillin, established beyond doubt that substances of microbial origin, in other words antibiotics, can find an important place as chemotherapeutic agents.

It is of special interest to draw attention here to the fact that three groups of investigations, which have laid the basis for the recent advances in our knowledge of antibiotics, dealt with the three groups of micro-organisms that are now considered as the most important producers of antibiotic substances, namely, the bacteria, the fungi and the actinomycetes:

(1) The work of Dubos (1939) on the tyrothricin complex produced by *Bacillus brevis*. This not only served to focus attention upon an important group of antibiotics, which are polypeptides in nature, but also laid the foundation for extensive studies of aerobic spore-forming bacteria which later resulted in the isolation of a large number of compounds, designated as 'subtilin', 'bacitracin', 'licheniformin', 'polymyxin', 'aerosporin', and many others.

(2) The work of the Oxford group, headed by Florey and Chain (Chain, Florey, Gardner, Heatley, Jennings, Orr-Ewing & Sanders, 1940; Abraham, Chain, Fletcher, Gardner, Heatley, Jennings & Florey, 1941) on penicillin. This was followed by numerous studies on penicillin-producing fungi, as well as on the general problem of fungi as organisms producing a large number of antibiotics. These studies resulted in the isolation of a large number of antibiotic substances including a variety of penicillins; none of the other antibiotics had so far shown any outstanding promise as chemotherapeutic agents.

(3) Our investigations (Waksman & Woodruff, 1940; Waksman, Horning, Welsch & Woodruff, 1942) on antibiotics produced by actinomycetes. Beginning with actinomycin—a highly toxic compound—this led to the isolation of streptothricin and finally of streptomycin, a highly important chemotherapeutic agent.

These studies were later followed by numerous investigations which resulted in the isolation of a large number of other antibiotics, some of which offer definite promise of finding practical applications in disease control (Table 1).

Table 1. *Classification of antibiotics of actinomycetes*

A. Soluble in ether and in other organic solvents:
- I. Pigmented substances:
  1. Orange coloured; somewhat soluble in neutral aqueous solution; nitrogen-bearing ring compound, highly toxic, $C_{41}H_{56}O_{11}N_8$; largely active against Gram-positive bacteria Actinomycin
     - *a*. Related compounds Actinoflavin
  2. Red-blue pigment; soluble in aqueous alkaline solution; active against Gram-positive bacteria Litmocidin
  3. Orange coloured; extracted from charcoal adsorbate and from mycelium by ether-alcohol mixture; active largely against *Mycobacterium tuberculosis* Nocardin
- II. Non-pigmented substances:
  1. Organic base; soluble in acidified aqueous solution, inhibits mostly Gram-positive bacteria Proactinomycin
  2. Largely fungistatic, not bacteriostatic, $C_{27}H_{42}N_2O_7$ Actidione
  3. Insoluble in water, present in mycelium; active largely against Gram-positive bacteria Ether-soluble factor (Streptocin)
  4. Neutral compound; slightly soluble in water, readily soluble in organic solvents, contains nitrogen (8·6%) and non-ionic chlorine (21·7%); active against various bacteria and rickettsiae Aureomycin, Chloromycetin

B. Insoluble in ether, but soluble in other organic solvents:
- I. Violet-blue pigmented substance Mycetin
- II. Colourless, sulphur-containing substance Sulfactin

C. Soluble in water, insoluble in ether and in other organic solvents:
- I. Bases soluble in aqueous acid solution; removed from charcoal by acid alcohol; active against various Gram-positive and Gram-negative bacteria:
  1. Little activity against *Bacillus mycoides*, *Serratia marcescens*; active against Bodenheimer organism and fungi Streptothricin
     - *a*. Compounds closely related to streptothricin, but varying in toxicity to animals and showing quantitatively different antibiotic spectra:
       - (*a*) *Streptin*. (*b*) *Streptolin*. (*c*) *Lavendulin*. (*d*) *Actinorubin*. (*e*) *Antibiotic* 136.
  2. Active against *B. mycoides* and *S. marcescens*, little activity against fungi, no activity against Bodenheimer organism; glycoside (streptidine-streptobiosamine) Streptomycin complex
     - *a*. Constituents of streptomycin complex:
       - (*a*) Streptidine-streptobiosamine, $C_{21}H_{39}N_7O_{12}$ Streptomycin
       - (*b*) Mannose derivative of streptomycin Mannosido-streptomycin
     - *b*. Streptomycin-like materials:
       - (*a*) *Antibiotic* F. (*b*) *Streptomycin* II.

Table 1 (*continued*)

II. Removed from charcoal by neutral alcohol, soluble in neutral aqueous solutions; narrow antibiotic spectrum against certain Gram-positive and Gram-negative bacteria	Grisein
1. Grisein-like material, still narrower spectrum than grisein, mostly enteric bacteria	Antibiotic 5310
D. Proteins and polypeptides:	
I. Colourless preparation, possessing lytic properties against living Gram-positive bacteria and dead Gram-negative bacteria	Actinomycetin
1. Active fraction of actinomycetin	Actinozyme
II. Active largely against micrococci; lyses cell membrane	Actinomyces lysozyme
III. Combined with orange pigment; largely bacteriostatic against Gram-positive bacteria	Micromonosporin
E. Incompletely described agents:	
I. Active against both Gram-positive and Gram-negative bacteria, including mycobacteria, and against fungi	Mycomycin
II. Produced by reddish to vinaceous-coloured mutant of *S. griseus*, active only against Gram-positive bacteria	Sv factor
F. Unknown agents:	
I.	Smegmatis factor
II.	Antiphage factors
G. Agents not produced readily in liquid media; activity obtained only on agar streak	
I.	Insoluble factors

It is now generally recognized that, for a new antibiotic to qualify as a chemotherapeutic agent, it must answer certain definite requirements, the most important of which are, briefly, as follows:

(1) It must be selective in action against various organisms, and not be a generalized protoplasmic poison.

(2) It must have desirable antibacterial properties, that is, it must affect bacteria or other micro-organisms that are not subject to the action of another antibiotic, or it must be more potent or more effective than another which it is to replace.

(3) It must exert not only a bacteriostatic but also a bactericidal effect upon bacteria or upon other micro-organisms.

(4) It should not be reduced in antibacterial activity by body fluids, it should not be inhibited by substances present in the blood, and should not be destroyed by tissue enzymes.

(5) It must not be toxic or at least not too toxic when injected into animals in amounts sufficient to combat infections.

(6) It should not damage leucocytes in concentrations required to affect bacteria, and should not be injurious to the kidneys and to other body tissues.

(7) It should preferably possess certain desirable physical and chemical properties, such as solubility in water, and a certain degree of stability.

(8) It must be excreted readily, but not too rapidly, from the animal system and must not accumulate there and produce undesirable after-effects.

(9) It should be active in the treatment of specific infections, preferably such which are as yet not susceptible to the action of any known drug.

(10) The possible synergistic effect of a new antibiotic with one already in use must not be overlooked, even if it does not by itself form an ideal chemotherapeutic agent.

In laying the foundation for the isolation of new antibiotics possessing desirable chemotherapeutic potentialities, numerous laboratories have made important contributions. Bacteriological and mycological laboratories contributed not only numerous cultures that produce new antibiotic substances, but also new strains of known cultures, which are more efficient producers of known antibiotics or new forms of such antibiotics. Chemical laboratories made important contributions in isolating and purifying these antibiotics, in freeing them of toxic or other undesirable impurities, and in separating from one another different antibiotics existing in mixture in the metabolite solution. The pharmacologist contributed to the progress of antibiotics and the recognition of their chemotherapeutic potentialities by his study of the behaviour of antibiotics in the animal body, especially their toxicity and *in vivo* activity. The physiologist and the biochemist made their contributions by studies of the mode of action of antibiotics upon bacteria. Finally, the clinician was ready to test the effectiveness of newly isolated antibiotics in the human body. By that time, if the new antibiotics were found to offer marked promise as potential chemotherapeutic agents, the chemical engineer and the manufacturer were ready to undertake production on a pilot and later on a factory scale.

The various steps outlined above did not necessarily follow in such sequence. Frequently, they ran parallel or overlapped one another. Usually, the physiologist and the biochemist came last. In the case of streptomycin, for example, less than a year passed between isolation of the antibiotic and its clinical use. This rapid progress was due primarily to three factors: (*a*) the great need for an agent that would affect bacteria not sensitive to penicillin; (*b*) the fact that the various laboratories were well geared to undertake an immediate study of a new and promising agent; (*c*) the fact that all the groundwork was laid previously by streptothricin, and the new antibiotic could fit into its groove, as regards general antibacterial activity, production, and isolation.

IV. PRESENT STATUS OF ANTIBIOTICS

Even a cursory examination of the investigations being carried out on the new antibiotics suffices to emphasize the tremendous amount of attention now being given to this new field of science. These studies are being made in laboratories devoted to microbiology and to chemistry, in pharmacological laboratories devoted to the study of infections, and in laboratories that may be designated as botanical, physiological, and biochemical. These laboratories comprise those of universities and government institutions, of hospitals and industrial organizations, of individual investigators, and of large co-operating groups. One is bewildered by the many cultures of micro-organisms, which may exceed tens of thousands in certain individual laboratories, that are being tested, and by the many new substances that are being continuously isolated. Frequently, the results obtained are published as soon as possible; often, there is considerable delay in publication due to the nature of the laboratories where such studies are made.

One begins to appreciate, therefore, the answer of one botanist, who, when asked a year or so ago how many laboratories were then engaged in the study of antibiotics, replied: 'All of them.' When one considers the numerous organisms that have already been isolated from various soils, water basins, composts, or dusts or simply taken from culture collections and tested for their antibacterial, antifungal, antiprotozoan, antiviral, and antitissue effects, one is surprised that many more substances have not been isolated or described. It is, of course, only too true that in order to gain priority many substances are named, but only insufficiently described, without awaiting their final isolation and test for purity. Since such descriptions are frequently based upon crude preparations, and since many organisms are now known to be capable of producing more than one antibiotic, much confusion has frequently resulted.

It may be of interest to examine, first, the various groups of micro-organisms as potential producers of antibiotics, and to summarize briefly our present knowledge of the properties of some of these antibiotics, before an attempt is made at broad generalizations and plans for future investigations.

1. *Types of antibiotics*

As this is written, less than 12 months have elapsed since the publication of the second edition of the book on microbial antagonisms (Waksman, 1947*a*), in which fifty-six antibiotics, either isolated or well recognized, were listed. Within this brief period more than three dozen new names have been added, including antibiotics produced by fungi, such as biformin, enniatin, geodin, pleurotin and tardin; by bacteria, namely, aerosporin, asterosporin, licheniformin, nisin and polymyxin; and by actinomycetes, such as actinorubin, actinozyme, chloromycetin, lavendulin and streptolin.

The complex biological potential of micro-organisms can be brought out no better than by an examination of their ability to produce antibiotic substances. Micro-organisms vary greatly in this respect. Certain groups contain a large proportion of forms possessing antagonistic properties, as in the case of the members of the genera *Penicillium* and *Aspergillus*, among the fungi, and of the genus *Streptomyces* among the actinomycetes; other groups, however, contain very few forms which are capable of inhibiting the growth of other organisms, as in the case of the Mucorales among the fungi. Some micro-organisms have limited antagonistic properties and their antibiotic potential is characterized by a very narrow spectrum, which may be directed against only certain few groups of bacteria, such as enterobacteria or mycobacteria (as in the *Mycobacterium smegmatis* or nocardin factor); other organisms have a broad antibiotic spectrum, which is active against numerous bacteria and even fungi, as in the case of clavacin. Some of the antibiotics inhibit the growth of bacteria only in very high concentrations, as in the case of nocardin, kojic acid or citrinin, which contain only 1000–50,000 units of activity per gram; others, however, are capable of inhibiting bacterial growth even in very low concentrations, as in the case of penicillin or actinomycin, with 100 million units per gram.

A broad survey of the antibiotic potentialities of micro-organisms leads to recognition of certain gradations in the types of antibiotics produced and in their complexity, as regards both the organisms producing them and the organisms sensitive to them. These gradations or stages are outlined here as follows:

(1) Some micro-organisms, notably many of the fungi, produce relatively simple antibiotics. It is sufficient to illustrate this by some of the compounds which are made up of only the elements carbon, hydrogen and oxygen; this is true, for example, of kojic acid ($C_6H_6O_4$), which is produced by the *Aspergillus oryzae* group, and clavacin, which is produced by *A. clavatus* and a variety of other fungi. These possess either only very limited antibiotic action, as in the case of the first, or have the properties of broad protoplasmic poisons, as in the case of the second.

(2) One may proceed from the above organisms to those which produce more than one antibiotic substance. This is true of various fungi and of certain actinomycetes. *A. fumigatus* is capable of producing, in addition to clavacin, also fumigacin, fumigatin, spinulosin, and possibly other compounds, as indicated by its antitissue properties. In addition to a variety of penicillins, the relative concentration of which depends on the strain of the fungus, the composition of medium, and conditions of cultivation, *Penicillium notatum* produces notatin and various antidotic and enhancement factors. *Streptomyces griseus* produces, in addition to two forms of streptomycin, at least two other antibiotics, one of which is active largely against fungi and has been designated as 'actidione', and another, active only against Gram-positive bacteria, which is present in the mycelium.

(3) Other micro-organisms, found largely among the bacteria, produce a still greater complexity of antibiotics. Two illustrations will suffice: (*a*) *Pseudomonas aeruginosa*, which yielded the first antibiotic ever studied, continues to give rise to new antibiotics; in addition to the older pyocyanase, pyocyanin and hemipyocyanin, there have recently been added to the list pyolipic acid and the pyo-compounds. (*b*) The *Bacillus subtilis* group is apparently capable of producing no end of antibiotics, as one would judge from the various compounds described under the names of 'subtilino', 'subtiline', 'subtilin', 'bacitracin', 'bacillin', 'subtilysin', 'eumycin', 'licheniformin' and 'trypanotoxin'.

(4) As one proceeds upwards in the scale of complexity of antibiotic mechanisms

one encounters a type of organism that produces a bewildering array of such substances. The best example of this is *Escherichia coli*. Not only are the different strains of this organism capable of forming antibiotic agents which are characterized by different antibacterial spectra, as shown by their action on other strains of the same organism and upon species of *Shigella* and of *Salmonella*, but these antibiotics vary also in their filterability, in their sensitivity to various temperatures, and in certain other properties.

(5) Finally, attention may be directed to those numerous micro-organisms which possess an antibiotic potential that is still very little understood, since no antibiotic can be demonstrated in liquid media upon which the organisms are grown. This is best illustrated by the fact that many actinomycetes exert a marked growth-inhibiting effect upon various bacteria when tested by the agar streak method, but when grown in liquid media no soluble substances can be demonstrated.

One may thus proceed from clavacin, an antibiotic which has the widest antimicrobial spectrum yet known, to substances, such as the *Escherichia coli* antibiotics, which are active only against other strains of *E. coli* and possibly a few other enteric bacteria. One may also proceed from kojic acid, with a relatively simple formula of $C_6H_6O_4$, through the more complicated nitrogenous forms, such as streptomycins ($C_{21}H_{37}O_{12}N_7$) and penicillins ($C_9H_{11}O_4SN_2.R$), and the chlorinated forms, such as ustin ($C_{19}H_{15}O_5Cl_3$) and chloromycetin to the various polypeptides, such as gramicidin and subtilin, and the complex proteins, such as the colicins and actinomycetin.

The various micro-organisms producing antibiotic substances frequently present large and variable groups. This is true, for example, of the *Penicillium notatum-P. chrysogenum* group producing penicillin and of the *Streptomyces griseus* group producing streptomycin. Although most of the strains of the former are apparently capable of producing some penicillin, only very few strains have been found capable of producing this antibiotic in large enough quantities to justify the present large-scale exploitation of this group.

The numerous cultures of *S. griseus* which have been studied in the laboratories of the Department of Microbiology of the New Jersey Station, either freshly isolated from soils or other substrates or obtained from other laboratories, have been divided into four groups: (1) those that produce streptomycin or streptomycin-like substances. (2) Those that produce grisein or grisein-like materials. (3) Those that form one or more antibiotics which are active only against certain Gram-positive bacteria, but not against Gram-negative organisms, thus distinguishing them from both streptomycin and grisein which are also active against certain Gram-negative bacteria; this group includes one mutant characterized by a vinaceous-coloured vegetative mycelium and obtained from the streptomycin-producing *S. griseus*, and the 1915 isolate of *S. griseus* deposited in the Baarn collection. (4) Those that produce no antibiotic substance. Most of the cultures of *S. griseus* thus far isolated at random belong to this group. They include also a mutant producing no aerial mycelium and obtained from the streptomycin-producing *S. griseus*, and the 1915 isolate of *S. griseus* kept in the New Jersey collection.

Numerous problems have arisen in connexion with the study of antibiotics. It is known, for example, that the effect of the medium or the substrate upon the antibacterial activities of an antibiotic varies greatly. Some substrates have no effect at all; others, because of the neutralizing action of some constituent, such as peptone, glucose, or serum, reduce the activity of the antibiotic; some substrates, such as blood, may inactivate completely certain antibiotics such as chaetomin. These facts have a bearing upon the variations among antibiotics in their toxicity to animals; some are extremely toxic and others have virtually no toxicity at all. Among the other problems of great importance in connexion with the utilization of antibiotics as chemotherapeutic agents, that of resistance deserves careful consideration. Bacteria sensitive to a given antibiotic may gradually develop resistance to it, if allowed to remain in contact with it for any length of time; some antibiotics favour the development of resistance much more readily than others.

These properties explain why, out of a hundred or more antibiotics now known, only very few have found practical application in chemotherapy. These may be listed as follows: (1) The antibacterial polypeptides, to which belong the tyrothricin complex, containing gramicidin, tyrocidine, and the closely related gramicidin-S; bacitracin and subtilin; as well as various other bacterial polypeptides, many of which are still not sufficiently defined. (2) The penicillin group of antibiotics, comprising the various forms now known as F, G, K and X. (3) The streptomycin complex. Although so far only two compounds have been found to belong to this group, there is no doubt that others will be isolated, perhaps more potent or with a greater variety of activities than streptomycin. These three groups of compounds have found wide application in the treatment of a great variety of infections caused by bacteria and other micro-organisms.

Great progress has been made in the production of these antibiotics. The yield of penicillin has been increased, through the isolation of new strains of penicillin-producing organisms, from one or two units to nearly 1000 units per millilitre of culture medium; the various forms of this antibiotic have been isolated and found to differ greatly in their antibacterial and in their *in vivo* effects. The potency of streptomycin has also been greatly increased, from 100 units to nearly 1000 units per millilitre, through the selection of strains and improvements in media; the antibiotic itself has been purified, and its cost greatly reduced. Certain elements were found to play specific roles in the production of certain antibiotics; it is sufficient to mention the role of zinc in the production of penicillin, of potassium in streptomycin, of iron in aspergillic acid and grisein, of manganese in polymyxin, bacillin, etc.

Numerous problems continue to attract the attention of the student of antibiotics. Rapid progress is being made daily, with continual shifting emphasis upon various aspects of the subject as new facts are uncovered and new ideas presented. Attention is directed here to some of the more outstanding phases of the field of antibiotics.

2. *Isolation of new antibiotics*

Various groups of micro-organisms have at one time or another received attention as potential producers of antibiotic substances. All of them are being reinvestigated at present. Substances that have long been buried in the literature are being reexamined and their antimicrobial behaviour determined. These organisms include (*a*) bacteria, (*b*) fungi, (*c*) actinomycetes, (*d*) algae, (*e*) Protozoa and other invertebrate animals. Although higher plants and higher animals produce a variety of substances which are similar in their antimicrobial properties to the true antibiotics, they may be considered as antibiotic-like in nature and need not be considered here in detail. It is sufficient to mention, among the plant products, some of the phytoncides, namely, allicin, protoanemonin, raphanin and tomatin, and, among the animal products, lysozyme, erythrin and lactenin.

Antibiotics of bacteria. Bacteria continue to receive considerable attention as producers of antibiotic substances. Among the previously isolated substances some have been investigated in detail. Bacitracin and subtilin, and possibly nisin, aerosporin and polymyxin, are promising antibiotics. Some have been manufactured on a limited scale. Their low toxicity, their high antibacterial activity, the effectiveness of some against Gram-negative bacteria and of others against *Mycobacterium tuberculosis* make them particularly interesting. A brief summary of our present knowledge of some of these recently isolated antibiotics will, therefore, be of interest.

Aerosporin is a basic substance, the methods of extraction being similar to those of streptomycin. It is active against Gram-negative bacteria, *Escherichia coli* being used as the test organism (Ainsworth, Brown & Brownlee, 1947).

Polymyxin is a basic substance soluble in water. It is highly active against various Gram-negative bacteria, both *in vitro* and *in vivo*; its activity is not affected by reaction of medium within a range of pH 5–8 (Stansley & Schlosser, 1947). It appears to be identical with aerosporin.

Bacitracin is a neutral compound, soluble in water and in organic solvents. Its polypeptide nature has recently been questioned. It is highly active against certain Gram-positive bacteria, has very limited toxicity to animals, and exerts, clinically, a marked effect in the treatment of infections caused by sensitive bacteria (Anker, Johnson, Goldberg & Meleney, 1948; Meleney & Johnson, 1947).

Licheniform was isolated from *Bacillus licheniformis*, a strain of *B. subtilis*. It is a highly nitrogenous basic substance, active against both Gram-positive and Gram-negative bacteria, including *Mycobacterium tuberculosis* (Callow, Glover & D'Arcy Hart, 1947).

Nisin is produced by certain lactic acid streptococci. It is active, both *in vitro* and *in vivo*, against various Gram-positive bacteria, including *Mycobacterium tuberculosis* (Mattick & Hirsch, 1947).

Subtilin is insoluble in 95% alcohol but soluble in 70%. It is active only against Gram-positive bacteria, including *Mycobacterium tuberculosis*, both *in vitro* and *in vivo.* It is a peptide and is sensitive to the action of proteolytic enzymes (Lewis, Feeney, Garibaldi, Michener, Hirschmann, Traufler, Langlykke, Lightbody, Stubbs & Humfeld, 1947).

Bacillin is formed by a large number of bacterial isolates from the soil. Forty-six cultures of bacilli isolated by Rudert & Foter (1947) from twenty-three soils produced this antibiotic. It is stable and is inactivated by H_2S; its activity is reduced in complex organic media, such as brain-heart infusion; it is insoluble in organic solvents; it is adsorbed on active carbons and partly eluted with aqueous ethanol; it is characterized by a wide antibacterial spectrum; adsorption on cation resin, followed by elution with pyridine gave 50-fold increase in activity; lyophilization destroys its activity.

Colicins are produced by certain enteric bacteria (Gratia & Frederick, 1946; Heatley & Florey, 1946; Frederick & Levine, 1947). They are highly specific in their action upon other enteric bacteria, the members of the genus *Shigella* being most sensitive and *Aerobacter* and Proteus least. The various colicins differ in their antibiotic spectra and in their physiochemical properties. Some strains of bacteria produce several colicins with different antibiotic spectra. They are peptides in nature and are destroyed by proteolytic enzymes; they are soluble in water, insoluble in organic solvents, and heat-stable. Their potential chemotherapeutic value is questionable.

Numerous other bacteria were found to have the capacity of producing antibiotic substances, but most of them, as in the case of the marine bacteria (Rosenfeld & ZoBell, 1947) have been but little studied.

Among the older antibiotics, some continue to receive attention, either because of new potentialities uncovered, as in the case of the tyrothricin complex, which has been modified chemically to yield derivatives which are less toxic and less haemolytic. In some cases, new mechanisms are discovered, as in the case of the antibiotics of *Pseudomonas aeruginosa*, which was found to yield new types of antibiotics such as the pyo-compounds and pyolipic acid.

Antibiotics of fungi. The fungi continue to receive their share of attention as producers of antibiotics. A number of new substances have been recently isolated. The possibility of utilizing fungi as producers of agents active against tumour cells (Kidd, 1947) is also of interest.

It is sufficient to mention several forms of enniatin, fructigenin, geodin, glutinosin, griseofulvin, which were found to be identical with the curling factor (Grove & McGowen, 1947), javanicin, tardin, biformin, lactaroviolin, lateritin, and pleurotin.

Ettlinger (1946) and Kavanagh (1947) published detailed reviews, and Brian & Hemming (1947), Hervey (1947), Wilkins (1947) and others have made detailed surveys of fungi.

One of the *Phycomycetes* yielded a product which is active against *Trypanosoma equiperdum*; it is soluble in organic solvents and in water. It has not been isolated, however, in a pure state, and its true antibiotic nature still remains undetermined (Schatz, Magnuson, Waksman & Eagle, 1946).

The Basidiomycetes have yielded a number of products, some of which, notably polyporin (Bose, 1947) and clitocibin (Hollande, 1947), were believed to offer potentialities as chemotherapeutic agents, the latter against *Mycobacterium tuberculosis*. These results have not been fully confirmed as yet, and the reported activity is not so high as that of other antibiotics that have already found chemotherapeutic application.

The Hyphomycetes have yielded a number of antibiotics, many of which are toxic and offer, therefore, little promise of becoming chemotherapeutic agents; the potentialities of others have still been little investigated. Among the more important agents, the penicillins occupy, of course, the leading place. The literature of this group of antibiotics continues to mount. Although the greatest interest is obviously manifested in the clinical application of the penicillins, the physiology of *Penicillium notatum* and *P. chrysogenum* and the biochemical problems concerned in penicillin production, as well as the mode of action of penicillin upon bacteria, are gradually being elucidated.

Various species of *Aspergillus* have also been found to produce penicillin. Some form, in addition, other antibiotics, such as aspergillic acid.

In addition to the genera *Aspergillus* and *Penicillium*, other genera among this group of fungi received attention as producers of antibiotics. It is sufficient to mention, in addition to the genera *Trichoderma* and *Chaetomium*, *Fusarium*, which was found to yield several antibiotics. The ability of members of the genus *Fusarium* to produce in artificial media substances toxic to other micro-organisms has long been known, beginning with the early work of Boyle and Pratt on staling. These organisms produce a plurality of antimicrobial agents, some of which, like javanicin, have been shown to possess antibacterial properties and a marked suppressive effect upon seed germination (Arnstein, Cook & Lacey, 1946). Among the other antibiotics isolated from Fusaria (Plattner, Nager & Boller, 1948), it is sufficient to mention enniatin A ($C_{24}H_{24}O_6N_2$) and enniatin B ($C_{22}H_{38}O_6N_2$); the first is considered as identical with lateritin. Whether the antibiotic isolated by Farmer (1947), which was given the formula $C_{26}H_{46}O_7N_2$, belongs to this group remains to be determined. The nutrition of the organism influences the nature of the antibiotic produced (Texera, 1948).

Of particular interest is the activity of some of the antibiotics of fungi upon *Mycobacterium tuberculosis*. It has been found that certain agarics (Ehrenberg, Hedstrom, Lofgren & Tankman, 1946) have a marked effect upon this organism. The pigment lactaroviolin (Willstaedt & Zetterberg, 1946) is active in very low concentrations. Clitocybin (Hollande, 1947) has attracted considerable attention in France. Some of the antibiotics isolated from lichens, such as usnic acid (Stoll & Renz, 1947) and ramularin (Marshak, 1947), have also been found to be highly effective against the tubercle organism not only *in vitro* but also *in vivo*. The substance isolated by Farmer (1947) inhibited the growth of the tuberculosis organism in dilutions of 1:160,000 to 1:640,000. A number of other fungus products have been found to be active against the tubercle organism. These have been reviewed in detail elsewhere (Waksman, 1947*d*).

Antibiotics of actinomycetes. Among the various groups of micro-organisms which are now being investigated as potential producers of antibiotics, the actinomycetes occupy a prominent place. Literally tens of thousands of cultures of actinomycetes are being isolated and tested in numerous laboratories throughout the world. This is due to the ease with which these cultures can be obtained from soils, manures, peats, river mud, dust and other natural substrates; to the large proportion of cultures (10–50%) that can be shown by simple methods of testing to possess antibacterial properties, and to the great practical potentialities of some of the antibiotics produced by these organisms, as shown to be the case for streptomycin.

Burkholder (1946) reported that out of 7369 cultures of actinomycetes isolated from the soil and tested by the agar-streak method, 1869 inhibited the growth of *Staphylococcus aureus*, 261 inhibited *Escherichia coli*, and 514 inhibited *Candida albicans*. Among the four genera of the actinomycetes, the genus *Streptomyces* has yielded the largest number of antibiotics. This is brought out in Table 1.

The streptomycin complex has recently been shown to be made up of two chemical entities, namely, streptomycin, which is chemically *N*-methyl-L-glucosaminido-streptosido-streptidine, and mannosido-streptomycin, which can be described chemically as D-mannosido-*N*-methyl-L-glucosaminido-streptosido-streptidine. Whether the streptomycin-like antibiotics which are produced by organisms other than *Streptomyces griseus*, such as *S. bikiniensis* (Johnstone & Waksman, 1948), are made of one or both of these chemical entities still remains to be determined. The specific entity of the streptomycin produced by certain organisms in admixture with streptothricin, such as antibiotic F and antibiotic 136, is still to be established.

Among the various species of *Streptomyces*, *S. griseus* and *S. lavendulae* have so far been found to be most important. These are large, heterogeneous groups of organisms, each of which has yielded a number of antibiotic substances.

For the isolation of streptomycin-producing strains from natural substrates, advantage has been taken of the resistance of such strains to streptomycin and of their sensitivity to actinophage to develop a suitable media (Waksman, Reilly & Harris, 1947).

The streptomycin-producing strains of *S. griseus* give rise to mutants, some of which do not form any streptomycin but which may produce other antibiotics, and others which form no antibiotics at all.

The *S. lavendulae* group is also widely distributed in nature and comprises a number of organisms capable of yielding a variety of antibiotics. In addition to the original streptothricin, other compounds are produced, some of which are more toxic to animals and others of which appear to be less toxic. The various cultures of *S. lavendulae* show variations in the amount of lavender pigmentation of their aerial mycelium on different media, as well as in other cultural properties, such as intensity of formation of soluble brown pigment, and morphological properties, such as structure of aerial hyphae. The antibiotics produced by members of this group vary greatly in their chemical composition, quantitative antibacterial spectra and toxicity to animals.

The chloromycetin-producing group of actinomycetes is also widely distributed, since a number of different cultures have now been isolated. In addition to the first reported by Ehrlich, Bartz, Smith, Joslyn & Burkholder (1947), Gottlieb, Bhattacharyya, Anderson & Carter (1948) isolated a culture from an Illinois soil which produced an antibiotic identical with chloromycetin. This substance is only slightly soluble in water, and its toxicity is comparable to that of streptomycin. It is inactive against yeasts, fungi and Protozoa, is moderately active against Gram-negative bacteria and *Mycobacterium tuberculosis*, and is highly active against Gram-negative bacteria, *Borrelia recurrentis* and *Rickettsia prowazeki*. It can be administered in propylene glycol solutions and orally (Smith, Joslyn, Gruhzit, McLean, Penner & Ehrlich, 1948).

Among the other antibiotic potentials produced by actinomycetes, the ability of some to interfere with the action of bacteriophages is of considerable interest, since it has a possible bearing upon the formation of antiviral agents (Schatz & Jones, 1947).

Antibiotics of other micro-organisms. The antibiotic potential of other micro-organisms has either not been sufficiently investigated or is still in dispute. The algae, for example, aside from the production of chlorellin, have so far not contributed anything of great interest. The trypanosomes have contributed the KR factor, which appears to have a marked effect upon tumours (Hauschka, Saxe & Blair, 1947). Other Protozoa are known to have marked capacities for digesting bacteria, but to what extent they are able to produce true antibiotics still remains to be determined. Comparatively little is known of the capacity of myxobacteria to produce true antibiotics. The ability of Protozoa to form a substance, designated as 'paramecin' which has the capacity of killing other Protozoa has also been established. Paramecin is an unstable compound, approaching in its inactivation enzymes and proteins (Van Wagtendonk & Zill, 1947).

3. *Antibiotics as chemotherapeutic agents*

Among the practical applications of antibiotics, none is of greater importance than their utilization in the treatment of infectious diseases in men and in animals. Unfortunately, many of the antibiotics thus far isolated have proved to be general protoplasmic poisons, which either affected directly the tissues of the host or produced other undesirable reactions upon the tissues or body fluids. Other antibiotics, which appeared very promising as a result of the *in vitro* studies, proved to be, by animal experimentation, of little significance for therapeutic treatment, because they were inactivated by the body fluids or were fixed by the serum proteins or by other blood constituents, thus becoming ineffective. Still other antibiotics proved to be of limited value because of other undesirable properties, such as insolubility in water and in tissue fluids, which thus restricted their use to the treatment of local infections.

Only very few antibiotics are being utilized at present for the systemic treatment of certain generalized bacterial infections. These are limited largely to the penicillins and to streptomycin. Tyrothricin is used for localized infections. Bacitracin, subtilin, aerosporin, polymyxin, nisin and chloromycetin appear to offer definite promise either as supplements or as independent chemotherapeutic agents. Penicillin, tyrothricin and bacitracin are active largely against Gram-positive bacteria, whereas streptomycin is active against both Gram-positive and Gram-negative bacteria, as well as against acid-fast bacteria. Penicillin is more effective against spirochaetes and some of the larger viruses. Although similar in certain respects, these two antibiotics possess distinct and characteristic antimicrobial spectra; they differ in chemical composition in their mode of action on disease-producing and other bacteria, and in their effects upon the cells and tissues of the host.

On the basis of the available clinical information, the utilization of antibiotics in the treatment of various infectious diseases can be outlined as follows, the diseases being grouped in several distinct categories:

(1) *Diseases caused by Gram-positive bacteria and certain Gram-negative cocci.* The organisms causing these diseases are among the most sensitive to a number of antibiotics. The various forms of penicillin have found extensive application in the treatment of these diseases. Bacitracin has the capacity of attacking some of these infections in a highly efficient manner. The tyrothricin complex has found application in treating wound infections. Bacteria made resistant to penicillin or strains naturally resistant to this antibiotic may still be sensitive to some of the other antibiotics, notably to streptomycin.

(2) *Diseases caused by Gram-negative bacteria.* These bacteria are, for the most part, resistant to penicillin and to bacitracin, but they are sensitive to several other antibiotics. One of these, streptomycin, has already found extensive application in the treatment of diseases caused by these organisms. Aerosporin and polymixin are other promising agents. The possibility of utilizing the synergistic action of two antibiotics, or of an antibiotic such as streptomycin with a synthetic agent such as sulphadiazine, offers promise of meeting the danger of rapid development of resistance of some of the bacteria to streptomycin; this has been done successfully in the treatment of certain forms of brucellosis and certain other infections.

(3) *Diseases caused by mycobacteria.* Because of their peculiar characteristics, diseases caused by acid-fast bacteria have proved to be among the most resistant to chemotherapy. *Mycobacterium tuberculosis*, despite its high sensitivity to many antibiotics *in vitro*, can be attacked in the body only in a manner which involves selective penetration and selective interference with the metabolism of these bacteria. The discovery that streptomycin can be utilized in the treatment of tuberculosis has provided a great stimulus in the search for new antibiotics that possess similar properties. This has given hope that the control of this highly important group of diseases is finally within our reach. The fact that streptomycin is not alone in this respect is indicated by the latent potentialities of a number of other antibiotics, such as subtilin, nisin, clitocybin, pyolipic acid and others. The possible development of strains of *Mycobacterium tuberculosis* resistant to streptomycin suggested the supplementary use of a synergistic agent, such as promin or para-amino-salicylic acid.

(4) *Spirochaetal diseases.* Several antibiotics, notably penicillin, have a remarkable effect upon diseases caused by spirochaetes. The antisyphilitic activity of the different forms of penicillin and bacitracin were found to vary greatly with the method of assay (Eagle & Fleischman, 1948). The use of penicillin in the treatment of these infections appears to be superseding gradually the methods of treatment current before the advent of antibiotic therapy (Moore, 1946).

(5) *Rickettsial diseases.* A number of antibiotics are highly effective upon rickettsiae. The discovery of chloromycetin points to the probability of successful combating of these diseases.

(6) *Fungus diseases.* A number of antibiotics, namely, hemipyocyanin, gliotoxin, clavacin and streptothricin, are known to possess marked fungistatic and fungicidal properties. Undoubtedly one or more of these will in time find application in the control of some of the diseases caused by fungi.

(7) *Protozoan and other diseases due to animal forms.* No true antibiotic is now known to be effective against diseases caused by protozoa, although the ability of certain agents to affect trypanosomes and other protozoan forms has been established.

(8) *Virus diseases.* Although certain viruses, like psittacosis and lymphogranuloma, are sensitive to some antibiotics, notably chloromycetin, none of the true virus diseases are found to be susceptible, such as the agents of the common cold and poliomyelitis.

(9) *Tumours or foreign cell infections.* There is a large number of other infections, such as those caused by foreign cells, namely, the tumours, for which no effective antibiotic is known at the present time. Although such cells are subject to the action of various organisms and their products, namely, certain bacteria, such as *Sporosarcina ureae* (Cohen, Borsook & Dubnoff, 1947), certain trypanosomes such as *Trypanosoma cruzii* (Hauschka *et al.* 1947), or fungi of the *Aspergillus fumigatus* type capable of producing a gliotoxin-like material (Kidd, 1947), and although some of the tumours respond clinically to certain chemical treatments, no successful chemotherapeutic agents have so far been found among the antibiotics.

(10) *Plant diseases.* Certain antibiotics are capable of destroying various bacteria and fungi which are causative agents of plant diseases; this has naturally aroused interest in the possibility of utilizing some of these agents as methods of control. This should hold true particularly of bacterial diseases of plants, since some of these are highly sensitive to various antibiotics (Gilliver, 1946). Attempts have been made to utilize antibiotics, such as streptomycin, which are resistant to destruction by micro-organisms for the treatment of certain plant infections caused by the Gram-negative bacteria. The fact that the ploughing under of stable manures and green manures leads to the suppression of a number of plant parasites, such as root rots, has suggested that this is due to the favourable development of antagonistic organisms (Waksman, 1947*a*).

This brief summary serves to emphasize the tremendous progress made in recent years in the development of a new branch of chemotherapy, comprising the utilization of antibiotics, frequently referred to as 'antibiotic therapy'. This has posed certain problems, which must receive careful consideration. The most important of these are the development of bacterial resistance and the mode of action of antibiotics.

4. *The problem of resistance*

As pointed out above, one of the most characteristic properties of antimicrobial agents, especially those that find application in chemotherapy, is their selective action upon different cells, whether those of unicellular bacteria or those of the higher forms of life. This holds especially true for the antibiotics. The selective action is not limited to different genera or even to species, but applies also to different strains of the same species, and even to different individual cells with a given culture of a certain strain of a known species. Thus, within a single species, there is marked variation in sensitivity of various strains, some being many times more resistant to a given antibiotic than are other strains.

It is especially the variation in sensitivity of cells within a given culture that results in the development of resistance of the culture to the antibiotic. Its extent and the speed with which such resistance develops depend largely upon the antibiotic and upon the organisms concerned. Increasing utilization, in recent years, of penicillin and streptomycin for the control of a variety of bacterial infections has focused particular attention upon this phenomenon. These two antibiotics differ not only in their respective antibacterial spectra, but also in the ease with which bacteria develop resistance to them. Streptomycin allows the development of resistance at a much more rapid rate and by a much larger number of organisms than penicillin, and it loses such resistance also much more slowly.

Different strains of the same organism also show considerable variation in their sensitivity to the same antibiotic. Staphylococci show ranges of sensitivity to penicillin from 1 to 100. The sensitivity of different strains of *Mycobacterium tuberculosis* to streptomycin was found by Youmans (1945) to range from 0·095 to 0·78 μg./ml. This natural variation in sensitivity of a given organism to a certain antibiotic is of great practical importance from a chemotherapeutic point of view, since it influences the selection of the particular antibiotic for the treatment of a given infection, and the concentration of the antibiotic to be used in clinical practice.

In addition to the natural variation in sensitivity of bacteria, originally sensitive organisms become gradually more resistant or 'fast' to a given antibiotic when allowed to be in contact with it either in the test-tube or in the body of the host. This phenomenon is not new either in bacteriology or in chemotherapy. It has long been observed, for example, that upon repeated administration of a drug, there is a change in susceptibility to it of the infecting organism. This decrease in sensitivity may thus be of two kinds: (*a*) loss or reduction in sensitivity, namely, acquired resistance, by an organism that was originally sensitive to a given drug; (*b*) occurrence of naturally resistant strains within a species which is normally sensitive to the drug.

The Oxford group of investigators (Chain *et al.* 1940); Abraham *et al.* 1941, in their first report on the use of penicillin for disease control, noted a marked increase in resistance of *Staphylococcus aureus* to penicillin upon continued use of the antibiotic. This observation was soon confirmed by numerous investigators, and was found to hold true also for certain other organisms naturally susceptible to penicillin. Bacteria acquire resistance to penicillin when cultivated in media containing gradually increasing concentrations of the drug, provided these are kept below the level inhibiting bacterial growth. Miller & Bohnhoff (1947*a*), for example, have shown that the tolerance of *Meningococcus* to penicillin should be greatly increased. As a result of 147 transfers through rising concentrations of the antibiotic, sufficient resistance was attained to permit growth in media containing 5000 units per ml. of penicillin. The resistance thus acquired was not a permanent property of the strain, but was gradually lost upon further growth in penicillin-free media. The resistant strains showed morphological differences which distinguished them from the susceptible strains, although no antigenic differences could be observed; those differences disappeared upon cultivation on penicillin-free media. *In vivo* resistance paralleled *in vitro* resistance.

The development of resistance to penicillin is limited only to certain few bacteria, and tends to disappear in the absence of penicillin. This problem becomes far more important in the case of streptomycin. Here, as well, bacteria are characterized by a natural and acquired resistance. Of particular importance, in this connexion, is the development of resistance by *Mycobacterium tuberculosis* in the case of certain patients who have been treated for considerable periods of time with this antibiotic.

Variation in sensitivity of *Escherichia coli* to streptomycin was found to range from 0·3 to 3·0 μg./ml., with an average of 1 μg./ml. for nine strains. In seven strains of *Proteus vulgaris*, the variation was from 0·3 to 2·5 μg./ml. Similar variations were obtained for other bacteria. A strain of *Pr. vulgaris* made resistant to streptomycin (from 0·6 to 7 μg./ml.) showed only a slight increase in resistance to streptothricin, a closely related antibiotic, and none to clavacin, a totally different type of antibiotic (Waksman, Reilly & Schatz, 1945).

Penicillin and streptomycin differ in the degree with which the bacteria made resistant to them become sensitive again. Fastness of staphylococci to penicillin is not permanent; bacteria made resistant to penicillin lose such resistance when grown in penicillin-free normal broth, and become again susceptible to relatively low concentrations of penicillin. Naturally resistant strains remain resistant, however, for much longer periods. *Pr. vulgaris* once made resistant to streptomycin, however, retained such resistance for a long time. Although resistance to streptomycin also develops in steps similar to those for penicillin, a high degree may be obtained in one step, in two steps or in several steps, streptomycin favouring the origin of resistant strains much more rapidly than does penicillin.

There is a wide range in the rate of development of resistance among different strains of the same organism. Williston & Youmans (1947) have found, for example, that out of eighteen strains of *Mycobacterium tuberculosis*, fourteen strains developed definite resistance, whereas four strains showed only a slight increase in resistance when grown in media containing streptomycin.

In a comparative study of the development of resistance of a strain of *Staphylococcus aureus* to different antibiotics, Klimek, Cavallito & Bailey (1948) found marked resistance developed to penicillin and to streptomycin, intermediate resistance to pyocyanin and to gliotoxin, and none to aspergillic acid. When the organism was made resistant to penicillin (1–4 mg./ml.), its biochemical reactions were lost, the cells becoming pleomorphic and Gram-negative. When the organism was made resistant to streptomycin, its characteristic biochemical reactions were also suppressed, but there was no noticeable change in its morphology. Bellamy & Klimek (1948) have also shown that resistant strains of *Staph. aureus* lost the capacity of growing anaerobically and became strict aerobes.

Various explanations have been suggested for the development of resistance. These may be summarized as follows: (1) Sensitive cells in a given bacterial population are killed, thereby enabling the more resistant cells to grow selectively. (2) More resistant mutants are formed in a sensitive population of bacteria. (3) Acquisition of new enzyme systems or new metabolic activities permit the organism to survive in spite of the presence of the particular growth-inhibiting agent. Demerec (1945) reported that resistance of *Staph. aureus* to penicillin originates through a mutation mechanism, and that the antibiotic acts as a selective agent to eliminate the non-resistant members of the bacterial population; the degree of resistance can be increased by selection, this increase being more rapid with each selection step.

Certain treatments tend to reverse the effect of resistance or to prevent its occurrence altogether. It has been shown (Voureka, 1948), for example, that strains of penicillin-resistant bacteria can be made sensitive again when they are grown in association with other bacteria, either penicillin-sensitive or penicillin-insensitive, or with certain bacterial autolysates.

In the development of resistance by bacteria to streptomycin, Miller & Bohnhoff (1947*b*) found that the *Meningococcus* produced two types (A and B) of variants:

A appeared in small numbers in all concentrations of the antibiotic; it produced large yellow colonies which grew both in streptomycin-free and in streptomycin-containing media, its virulence for animals being similar to that of the original strain.

B appeared in greatest numbers in concentrations of streptomycin between 100 and 400 μg./ml., its colonies varying in size and colour, depending upon the concentration of the antibiotic; this type was dependent upon the presence of streptomycin in the medium; it did not grow in media containing less than 5 μg./ml. streptomycin, and it was non-virulent for mice, unless the mice received streptomycin.

These results were confirmed by a number of investigators for different bacteria. Paine & Finland (1948), for example, obtained from sensitive cultures of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Ps. morgani* both 'resistant' and 'dependent' variants. The critical concentrations of streptomycin above which the sensitive strain did not grow were about the same as those below which the dependent variant did not grow. These results tend to confirm the concept that antibacterial agents act as metabolite antagonists, streptomycin interfering with some essential metabolite or metabolic process of the sensitive strains and serving as a metabolite or growth factor for the dependent strain. This relation can be reversible for the dependent strains, but it is permanent in the case of the resistant strains.

These phenomena raise some very important problems in connexion with the use of streptomycin in chemotherapy.

5. *Mode of action of antibiotics*

The antimicrobial action of antibiotics is said to be primarily growth-inhibiting in nature, by interfering with cell multiplications; the cell, unable to divide, gradually dies. Antibiotics, however, also possess marked bacterial properties. The nature of the antibiotic, the age of the bacterial cell, the nature and composition of medium in which the organism grows, the environmental factors of growth, all influence the effect of the antibiotic upon bacteria. Most of these theories proposed to explain the mechanism of bacteriostatic and bactericidal activities of antibiotics are largely speculative in nature. Among these theories, the following deserve consideration:

(1) Antibiotics interfere with some of the metabolic processes of the microbial cell, by substituting for one of the essential nutrients. Substances that are structurally related to the normal cell nutrients may thus exert a specific inhibitory effect. These substances are taken up by the cell and block the natural processes of growth.

(2) Antibiotics affect the cell by interfering with the utilization of one of the intermediary metabolic products. Streptomycin has been found, for example, to have the capacity of blocking the metabolism of certain amino-acids by bacteria. This was shown by Geiger (1947) for the oxidation of amino-acids by *Escherichia coli*. Gale & Rodwell (1948) reported that when growing in presence of very high concentrations of penicillin, *Staphylococcus aureus* adopts a different form of amino-acid metabolism; resistant strains lose their capacity for concentrating free glutamic acid in the internal environment and are able to synthesize all their amino-acid requirements from ammonia and glucose in the presence of thiamin.

(3) Certain antibiotics interfere with various enzymatic systems, notably the respiratory mechanisms of the bacterial cell. Sevag (1946) believed that this type of inhibition is the basis for the mechanism of antibacterial action of sulphonamides and probably of antibiotics. These enzymes are concerned not only with oxygen uptake and production of acid by the cells, but also with the synthesis of essential metabolites and coenzymes. The phosphate uptake by bacteria accompanying glucose oxidation is another illustration of an enzyme mechanism that can be interfered with. The antibiotic may affect respiration only indirectly by competing with a metabolite for a certain enzyme system.

(4) An antibiotic may prevent the synthesis of some essential metabolite by the bacterial cell. The interference with the production and utilization of an essential growth factor required by the cell may be only one form of such interference.

(5) An antibiotic may combine with a sulphydryl group which is essential for cell multiplication.

(6) Interference with the utilization of iron by the bacterial cell or with the functioning of the iron-containing enzyme system, as in the action of aspergillic acid, has also been suggested.

(7) The antibiotic may act as a detergent and affect the surface tension of the bacterial cells.

(8) The activity of an antibiotic may be a function of several factors, such as diffusibility into the microbial cell, adsorption by various enzyme systems, reaction with the sulphydryl groups of enzymes or with sulphydryl-containing substances adsorbed by the enzyme.

(9) The prolongation of the lag phase, the reduction of the growth rate, the lowering of stationary population, and the hastening of death of the bacteria have also been considered as mechanisms of antibacterial activities. These effects are involved in the bacterial action of the antibiotic, whereas a bacteriostatic effect may consist in the selective action of the antibiotic upon one stage only.

Gale & Taylor (1946) postulated four successive effects of penicillin upon the assimilation of glutamic acid by *Staphylococcus aureus*: (*a*) the cells become non-viable, (*b*) cell respiration progressively fails, (*c*) lysis sets in, (*d*) assimilation of nutrients is prevented. The last effect may precede the second and third stages. In surveying the complex problems involved in the modes of inhibition of bacterial growth, Sevag (1946) concluded that this 'may indicate that bacteria do not thrive by means of rigid or straight-jacketed mechanisms, but that they exercise versatile metabolic activities'. The cytochemical evidence for the mode of action of penicillin on bacteria has recently been reviewed by Pratt & Dufrenoy (1948).

Garrod (1948) made a detailed study of the bactericidal properties of streptomycin. The rapidity and extent of this bactericidal action were found to depend upon: (*a*) concentration, high concentrations having rapid effect; (*b*) temperature, the bactericidal effect being accelerated by an increase in temperature, very little effect taking place at 5° C., and a much greater effect at 37° C. than at 28° C.; (*c*) strain or organism; (*d*) nature of medium, the effect being exerted in all nutrient media, including certain body fluids, but only to a light extent in non-nutrient media; (*e*) reaction of medium, an acid reaction being far less favourable than an alkaline reaction; (*f*) the size of the inoculum, a small population being destroyed rapidly, whereas in a large one many cells survive; (*g*) as compared to penicillin, the bactericidal action of streptomycin is more immediate, beginning, under favourable conditions, in 1 min. It was suggested that the therapeutic effects of streptomycin are due to its bactericidal rather than to its bacteriostatic action.

6. *Antibiotics in natural processes*

The ability of so many micro-organisms to produce agents that have the capacity of inhibiting and even of destroying other organisms, the fact that these substances vary so greatly in chemical composition and in their mode of action, and especially the fact that some of these substances have found extensive application as chemotherapeutic agents, have all tended to arouse the imagination of all those who are interested in interrelations among living systems in a natural environment and have served as a basis for numerous speculations. In a study of the factors underlying the survival of a microbe in an association of microbes, one may examine the processes that occur in the soil. When a microbe has been freshly introduced into the soil it has to compete there, in order to survive, with the numerous microbes that are already established in the soil. In other words, it has to fit in with the complex interrelations existing among the numerous micro-organisms that make up the complex population of the soil. The conditions which control the survival of such a hypothetic microbe may be outlined briefly as follows: (1) The ability of the particular organism to utilize more readily, for nutritive purposes, some of the inorganic or organic chemical constituents in the soil as compared to other micro-organisms inhabiting this substrate. (2) The ability of the organism to tolerate the specific conditions of reaction, moisture and temperature which prevail in a given soil. (3) The capacity of the organism to produce substances inhibitive to the growth of other organisms, notably organic acids, alkaline products and specific toxins or antibiotics. (4) The presence in the soil of a protective agent which the specific organism can utilize as against the other members of the soil microbial population; this is true, for example, of root-nodule bacteria which not only find in the roots of leguminous plants a suitable habitat but are also able to adjust their metabolic processes to those of the plant. Casas Campillo (1947) has shown that the great majority of spore-forming bacteria in the soil (42–69 % of the cultures tested) are antagonistic to root-nodule bacteria. When grown in liquid media, they produce antibiotic substances, which could be adsorbed on norit and removed with 95 % alcohol. This activity may play an important role in the reduction of nodule formation on leguminous plants.

In the case of plant pathogens, the presence of hosts will enable the pathogenic organism introduced into the soil to survive for a much longer period than in the absence of such hosts. Rudert & Foter (1947) have suggested, for example, that the production of bacillin by various spore-forming soil bacteria may play a role in controlling the microbial flora of the soil.

These facts hardly lend themselves to any possible interpretation concerning protective or combative mechanisms on the part of the bacteria and fungi concerned in their struggle for survival in the soil. Whether the production of specific metabolic products of the nature of antibiotics actually gives the organisms producing them certain advantages as regards available food and space is not known at present. The ability to form antibiotics does not supply additional nutrients to the organisms concerned, nor are the antibiotics thus produced responsible for the elimination of organisms competing for available space.

Without going into a more detailed discussion of the relationship of antibiotics to living processes among micro-organisms living in a mixed population in a natural environment, one is justified, on the basis of these cursory observations, in concluding that the phenomena of antibiotics and the production of antibiotics are accidental properties of certain organisms. These properties appear to be, in most cases, of limited importance to the organism producing them, in their competition with other organisms for survival or for food. These properties are taken advantage of for the production of substances that have the capacity for combating other organisms, notably disease-producing bacteria. It is to be remembered that such substances are produced in a highly artificial environment under special conditions of nutrition. In a synthetic medium or in a type of poor medium that one would encounter in the soil, such organisms would hardly have an opportunity to produce the antibiotics in question.

Numerous illustrations could be cited to substantiate the above assertions. Suffice to say that the present facts do not warrant any attempts to generalize concerning the importance of the capacity for producing antibiotics to the particular organisms in their struggle for survival and multiplication in a natural environment such as the soil.

Knowledge of the optimum conditions for survival of the pathogenic and saprophytic organisms makes possible the treatment of the soil which will favour the saprophytes to the detriment of the pathogens. This is true, for example, of the use of green manures for the control of the potato-scab organism in the soil. The suggestion that green plant materials favour the development of organisms which are antagonistic to the potato-scab organism was not confirmed by subsequent investigators. The results appear to be due rather to the production of some acidity or to an increase in the moisture content of the soil, both of which are unfavourable to the scab-producing organism. The ability of various organisms to produce external metabolites ('ectocrine substances') which have an important environmental significance both for the organisms producing them and for the other organisms in the community has been recently reviewed, with particular emphasis upon marine communities, by Lucas (1947).

V. THE FUTURE OF ANTIBIOTICS

As one surveys the broader aspects of the science of antibiotics, as one realizes that a mere fraction of the micro-organisms present in the numerous soil types throughout the world, in water basins, on food products, and on other substrates have so far been examined, as one visualizes the great variety of chemical compounds which are produced by these organisms and which have the capacity of inhibiting the growth of and even of destroying other micro-organisms, as one finds that many of the isolated compounds are not too toxic to animals and possess properties which would render them desirable chemotherapeutic agents, one is inclined to become optimistic and assert that, before long, all human ailments, and possibly also animal and plant diseases as well, will be combated if not completely eliminated.

On the other hand, as one realizes that of the numerous antibiotics so far isolated only two, penicillin and streptomycin, have become true chemotherapeutic agents, with only few others, notably tyrothricin, bacitracin, polymyxin and chloromycetin occupying important positions, and as one recognizes the rapid development of resistance among bacteria against some of these antibiotics after continued use, a fact which has become particularly striking in the treatment of certain urinary infections and certain forms of tuberculosis with streptomycin, one's optimism is somewhat reduced in regard to the future outlook of antibiotics.

An impartial examination of the facts reveals that there is no cause for undue optimism, and certainly there is little cause for undue pessimism. The rapid progress made in the development of penicillin and streptomycin, the fact that these two antibiotics have proved to be ideal chemotherapeutic agents with very limited toxicity, especially in the case of penicillin, and the great effectiveness of these two antibiotics against a large variety of bacteria are responsible for the tendency to become too expectant of immediate new discoveries. The fact that such discoveries are possible is indicated by the recent isolation of chloromycetin, which has great activity against the rickettsiae, the recent isolation of aerosporin and polymyxin, active against various Gram-negative bacteria, the isolation of a number of agents, such as subtilin and nisin, active against *Mycobacterium tuberculosis*, the various reports of the ability of certain products of different micro-organisms to attack tumour cells, and the ability of products of certain actinomycetes to attack bacterial viruses or phages.

The future will require, even more than the past, the co-ordinated efforts of the microbiologist, the chemist, the pharmacologist, the physiologist, and the clinician, in the isolation, testing and utilization of new antibiotics. It will require examination of numerous freshly isolated cultures of organisms, determining their antimicrobial potentialities under different conditions of culture and in media of different composition, selecting those organisms with more potent and more varied activities. With several antibiotics already established as important chemotherapeutic agents, the new antibiotics will have to be more effective, they will have to act on organisms resistant or not too sensitive to the known antibiotics, they will have to favour less the development of resistant varieties, they will have to be less toxic.

I have often been asked whether I and my collaborators succeeded in isolating streptomycin by observing an organism growing on a bacterial plate accompanied by the killing of such bacteria on the plate. My stock answer is something as follows: 'About 10,000 cultures of organisms have been examined. Of these, about 1000 were found to have antibacterial properties, when tested by the agar-streak method. When these were grown in liquid culture media, only about 100 were able to produce in the medium substances which had the capacity of inhibiting the growth of various bacteria and other organisms. Of these, ten substances have actually been isolated and described. Of these ten, one proved to be streptomycin.' These facts are of course very approximate, but they fit the story well. Actually the number of cultures tested in this laboratory, since 1936, for their ability to inhibit the growth of bacteria and other micro-organisms, and since 1939, for their ability to produce antibiotic substances, would probably approach nearer the figure of 25,000 than that given above. The proportion of cultures possessing antagonistic properties, especially among the actinomycetes, is nearer to 20% and may even reach 50% rather than the 10% figure mentioned above. Lack of sufficient chemical help prevented the isolation of larger numbers of compounds, and the rapid advances made by penicillin and streptomycin have made it impossible to focus more attention on some of the other antibiotics, such as streptothricin and grisein, which appear, on the basis of insufficient experimentation however, to have definite promise.

Certain important problems are now recognized and will no doubt form the basis for future investigations. Some of these may be briefly listed here:

(1) Need for the isolation of new antibiotics, more potent than those known at present, or possessing more desirable physical, chemical and biological properties, or which do not permit the rapid development of bacterial resistance.

(2) Need for antibiotics which are capable of exerting a synergistic effect when combined with other known antibiotics.

(3) A knowledge of the mechanism of the development of resistance, and the problem of overcoming such resistance by bacteria to certain antibiotics.

(4) An understanding of the mode of action of antibiotics upon bacteria.

(5) The isolation of antibiotics which are active against filterable viruses, and against abnormal body cells, such as tumours.

Some of the newer antibiotics show promise of becoming useful chemotherapeutic agents in themselves or as supplements to other antibiotics; some of these deserve careful consideration. Some of these antibiotics are characterized by wide antimicrobial spectra, others are more limited in their action against different bacteria. The width of the spectrum is no indication, however, of the chemotherapeutic potentialities of a substance. Although Chain & Florey (1944) were inclined to consider antibiotics with a wide spectrum ('antiseptics') as potentially toxic agents, whereas those with a narrow spectrum ('bacteriostatics') were looked upon as offering greater therapeutic possibilities, this has not always been substantiated. It is true, for example, that clavacin, which possesses a wider antibacterial spectrum than penicillin, is much more toxic. Comparison of streptothricin with streptomycin, however, shows the former to have a narrower antibacterial spectrum than the latter, as regards certain Gram-positive and Gram-negative bacteria, but streptothricin leaves a residual toxic effect in the animal body and streptomycin does not. Thus, of the two, the latter is the more desirable chemotherapeutic agent.

The ability of certain micro-organisms to produce agents which are active against bacterial toxins (antidotics), against bacterial phages and animal viruses (antivirotics), and against tumour cells has been definitely established. Little is known, however, of the mechanisms involved, and the possibility of utilizing such mechanisms for chemotherapeutic purposes.

The search for new antibiotics will continue. A few, perhaps only very few, will no doubt be found which will prove better than those now known, or which may act upon disease-producing agents not susceptible at present to chemotherapy. Some of them will act alone and others will contribute synergistically to the action of the antibiotics known at present. The problem of antiviral and antitumour agents will be investigated on a broader scale. Finally, a better knowledge of the physiological and biochemical mechanisms of the bacteria which are affected by the various antibiotics may help to clarify the still obscure aspects of the mode of action of antibiotics, and thus possibly lead to discovery or even to synthesis of new agents. The prospects for antibiotics are promising, the future is bright indeed.

VI. SUMMARY

1. The phenomenon of antagonism or antibiosis among micro-organisms dates back to the early days of microbiology. The early studies made by students of mixed infections, of accidental contaminations of cultures, of the effects of one organism or its metabolic products upon another, of the isolation of such products, and even attempts to utilize them for disease control, all represented a series of unco-ordinated observations rather than a system which would fit into a new and important branch of science. The new 'antibiotic age' dates only to 1938–9, when a series of co-ordinated studies were made in several laboratories throughout the world.

2. Antibiotics, known previously as lysins, toxins, lethal principles, staling principles, etc., are characterized by certain important properties which distinguish them sharply from the common antiseptics and disinfectants. They are produced by micro-organisms, they are selective in their action upon bacteria and other micro-organisms, they vary in their chemical and physical properties, they differ in their mode of action and are affected differently by the composition of the substrate in which they act, they vary in their toxicity to animals, and, therefore, in their chemotherapeutic potentialities.

3. Antibiotics are produced alone or in mixture by different groups of micro-organisms, notably bacteria, fungi and actinomycetes. Some thirty or more antibiotics have now been isolated from each of these groups. Among the bacteria, the spore-forming aerobes are the most important, although cocci, Gram-negative and other bacteria have been found to yield important antibiotics, nisin having been isolated from streptococci, and pyocyanase, colicins, and a variety of other compounds from the Gram-negative rods. Among the fungi, the *Penicillium* and *Aspergillus* groups are most important, although the *Trichoderma*, *Chaetomium* and other groups, as well as various Basidiomycetes have been found to yield interesting compounds. Among the actinomycetes, the genus *Streptomyces* is most important, although the genera *Nocardia* and *Micromonospora* have also been found to produce interesting antibacterial substances.

4. Out of more than 100 antibiotics that have so far been isolated, only very few have found application as chemotherapeutic agents. These are penicillin, streptomycin and tyrothricin. Among the other more promising antibiotics, one may include bacitracin, subtilin, licheniformin, nisin, aerosporin, polymyxin and chloromycetin.

5. The introduction of antibiotics as chemotherapeutic agents has revolutionized medical practice. It has pointed a way to the treatment of infections that did not lend themselves previously to therapy. It has given rise to optimistic expectations that new agents will be found that will lend themselves to the treatment of numerous diseases which are still resistant.

6. Among the present-day problems in the field of antibiotics may be listed the development of resistance among bacteria on prolonged contact with the drug, the search for synergistic agents which would tend to overcome such resistance, a better knowledge of the mode of action of antibiotics, and a search for new antibiotics, notably those that are active upon infections, such as virus and tumour infections, and that do not lend themselves at present to chemotherapy.

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